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INVESTIGATIONS INTO GENOMIC INSTABILITY OF ENDOGENOUS AND TRANSFECTED hprt GENES IN MOUSE AND HUMAN TUMOUR CELLS

By

Diana Wilkinson

A thesis submitted to
the school of Graduate Studies and Research
in partial fulfilment of the requirements for the degree of
Doctor of Philosophy

Department Microbiology and Immunology

University of Ottawa
Ottawa, Ontario
Nov, 1997

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ABSTRACT


A mouse model system was developed to sensitively detect mutagenic events arising spontaneously in mouse fibrosarcoma cells grown under *in vitro* or *in vivo* conditions. These cells were genetically manipulated to enhance their sensitivity as detectors of mutational events at the X-linked *hpert* (hypoxantine phosphoribosyltransferase) gene, a frequently used marker of genomic instability. A fibrosarcoma cell with three X-chromosomes and randomly distributed X-specific fragments was isolated from a male mouse. Initially, this cell line was resistant to mutation induction, presumably due to the presence of multiple *hpert* genes. Inactivation of all but one *hpert* gene resulted in a cell line with a heterozygous marker which was at least 1000 times more sensitive to detecting induced mutational events. Infection of this derived cell line with a selectable marker for neomycin resistance allowed recovery of cells which subsequently demonstrated that the tumour environment was capable of enhancing spontaneous *in vitro* mutation induction.

A human lymphoblastoid cell line was also genetically manipulated to establish nine stable transfected models for the study of genomic instability. The endogenous X-linked *hpert* gene was mutated, presumably by a large deletion, and subsequently transfected with a human *hpert* minigene encoding for its own promoter and polyadenosine signal. Transfected clones demonstrated a 2 to 600 fold increase in spontaneous mutation rate at the transgene loci compared to the endogenous *hpert* locus. There was no evidence of mutator phenotype activation in any of the clones as judged by testing at another locus, the endogenous *tk* (thymidine kinase) gene. An inverse correlation was also noted between the number of the inserted sequences and the stability of gene expression, with single gene insertions being most stable and tandem insertions being the least stable.
ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to the many people who contributed to the research in this thesis, and made my graduate training a rewarding experience.

First I would like to thank my supervisor, Dr. Chaim Birnboim, for many hours of advice and suggestions which enabled me to achieve my goal.

I am grateful to the members of my committee, Dr. D.H. Blakey, Dr. E. Brown, and Dr. R.G. Korneluck, for all the helpful advice throughout the years and Dr. M. Goddard for all statistical advice.

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Special thanks to my family whose love and patience was never ending.

Finally, I would like to extend my deepest gratitude to Dr. Jack McLean of Health Canada, my mentor, without whose everlasting support and enthusiasm I would not have been able to do any of this.
For Shelley and Tyler
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<tbody>
<tr>
<td>alu</td>
<td>human repetitive sequence</td>
</tr>
<tr>
<td>APRT</td>
<td>adenine phosphoribosyltransferase</td>
</tr>
<tr>
<td>aprt</td>
<td>adenine phosphoribosyltransferase gene</td>
</tr>
<tr>
<td>aprt&lt;sup&gt;+&lt;/sup&gt;</td>
<td>heterozygous at the adenine phosphoribosyltransferase gene locus</td>
</tr>
<tr>
<td>aprt&lt;sup&gt;−&lt;/sup&gt;</td>
<td>homozygous mutant at the adenine phosphoribosyltransferase gene</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>bp</td>
<td>base pair(s)</td>
</tr>
<tr>
<td>BSS</td>
<td>Hanks' balanced salt solution, Gibco BRL, Burlington, Canada</td>
</tr>
<tr>
<td>CCS</td>
<td>1 mM sodium citrate, 1 mM CDTA, 0.1% SDS - pH 6.8</td>
</tr>
<tr>
<td>CDTA</td>
<td>cyclohexane diaminetetraacetic acid</td>
</tr>
<tr>
<td>CHT</td>
<td>10&lt;sup&gt;−5&lt;/sup&gt; M deoxycytidine, 1x10&lt;sup&gt;−4&lt;/sup&gt; M hypoxanthine, and 1.5x10&lt;sup&gt;−5&lt;/sup&gt; M thymidine</td>
</tr>
<tr>
<td>CHAT</td>
<td>10&lt;sup&gt;−5&lt;/sup&gt; M deoxycytidine, 1x10&lt;sup&gt;−4&lt;/sup&gt; M hypoxanthine, 4x10&lt;sup&gt;−7&lt;/sup&gt; M aminopterin and 1.5x10&lt;sup&gt;−5&lt;/sup&gt; M thymidine</td>
</tr>
<tr>
<td>CHAT&lt;sup&gt;R&lt;/sup&gt;</td>
<td>deoxycytidine, hypoxanthine, aminopterin and thymidine resistant</td>
</tr>
<tr>
<td>c-H-ras-1</td>
<td>c-Harvey-ras oncogene</td>
</tr>
<tr>
<td>cox&lt;sub&gt;4&lt;/sub&gt;</td>
<td>cytochrome oxidase gene</td>
</tr>
<tr>
<td>CpG</td>
<td>GC rich regions of DNA (often methylation sites)</td>
</tr>
<tr>
<td>CT</td>
<td>10 mM Tris-hydrochloride, pH 7.5, 1 mM CDTA</td>
</tr>
<tr>
<td>D&lt;sub&gt;37&lt;/sub&gt;</td>
<td>dose that results in 37% survival</td>
</tr>
<tr>
<td>DAPI</td>
<td>4′,6′-diamidino-2-phenylindole</td>
</tr>
</tbody>
</table>
DES  deoxyribonucleic acid extracting solution

dhfr  dihydrofolate reductase gene

DMEM  Dulbecco's modified Eagle medium

FCS  fetal calf serum

FISH  fluorescence in situ hybridization

FITC  fluorescein-5-isothiocyanate

G418  geneticin

gpt  guanine phosphoribosyltransferase gene

Gy  gray (1 gray = 100 rad)

HAT  $1 \times 10^{-4}$ M hypoxanthine, $4 \times 10^{-7}$ M aminopterin and $1.5 \times 10^{-5}$ M thymidine

HAT$_r$  hypoxanthine, aminopterin and thymidine resistant

HAT$_s$  hypoxanthine, aminopterin and thymidine sensitive

HEPES  N-2-hydroxyethylpiperazine-N’-2-ethane-sulfonic acid

HPRT  hypoxanthine phosphoribosyltransferase

hppt  hypoxanthine phosphoribosyltransferase gene

HPRT$^-$  functional hypoxanthine phosphoribosyltransferase

HPRT$^-$  mutant hypoxanthine phosphoribosyltransferase

hppt$^{-}$  hemizygous for the hypoxanthine phosphoribosyltransferase gene

HT  $1 \times 10^{-4}$ M hypoxantine and $1.5 \times 10^{-5}$ M thymidine

kb  kilobase(s)

LTR  retroviral long terminal repeat promoter
<table>
<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>Mb</td>
<td>megabase(s)</td>
</tr>
<tr>
<td>MDM</td>
<td>modified Dulbecco's medium</td>
</tr>
<tr>
<td>MIP</td>
<td>methylation induced premeiotically mutation</td>
</tr>
<tr>
<td>MNU</td>
<td>N-methyl-N-nitrosourea</td>
</tr>
<tr>
<td>NaOAc</td>
<td>sodium acetate</td>
</tr>
<tr>
<td>neo</td>
<td>neomycin gene</td>
</tr>
<tr>
<td>NS</td>
<td>non-selective medium</td>
</tr>
<tr>
<td>p53</td>
<td>tumour supressor gene</td>
</tr>
<tr>
<td>PBS</td>
<td>Dulbecco’s calcium-magnesium-free phosphate buffered saline, Gibco BRL, Burlington, Canada.</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>pgk</td>
<td>phosphoglycerol kinase</td>
</tr>
<tr>
<td>NP-40</td>
<td>Nonidet P-40 detergent</td>
</tr>
<tr>
<td>PRPP</td>
<td>5-phosphoribosyl-1-pyrophosphate</td>
</tr>
<tr>
<td>rec</td>
<td>recombinase gene</td>
</tr>
<tr>
<td>RES</td>
<td>RNA extracting solution</td>
</tr>
<tr>
<td>RIP</td>
<td>repeat induced point mutation</td>
</tr>
<tr>
<td>RPMI 1640</td>
<td>human lymphoblastoid culture medium</td>
</tr>
<tr>
<td>RT</td>
<td>reverse transcriptase</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SPTG$^5$</td>
<td>mouse myeloma cell line sensitive to 6-thioguanine</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>SSC</td>
<td>standard saline citrate (0.15 M NaCl, 0.15 M sodium citrate)</td>
</tr>
<tr>
<td>TFT</td>
<td>trifluorothymidine</td>
</tr>
<tr>
<td>TFT&lt;sup&gt;R&lt;/sup&gt;</td>
<td>trifluorothymidine resistant</td>
</tr>
<tr>
<td>6-TG</td>
<td>6-thioguanine</td>
</tr>
<tr>
<td>6-TGMP</td>
<td>6-thioguanine monophosphate</td>
</tr>
<tr>
<td>6-TG&lt;sup&gt;R&lt;/sup&gt;</td>
<td>6-thioguanine resistant</td>
</tr>
<tr>
<td>6-TG&lt;sup&gt;S&lt;/sup&gt;</td>
<td>6-thioguanine sensitive</td>
</tr>
<tr>
<td>TK</td>
<td>thymidine kinase</td>
</tr>
<tr>
<td><em>tk</em></td>
<td>thymidine kinase gene</td>
</tr>
<tr>
<td>TK&lt;sup&gt;-&lt;/sup&gt;</td>
<td>functional thymidine kinase</td>
</tr>
<tr>
<td>TK&lt;sup&gt;+&lt;/sup&gt;</td>
<td>mutant thymidine kinase</td>
</tr>
<tr>
<td><em>ts</em></td>
<td>thymidylate synthase gene</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
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</tbody>
</table>
CHAPTER 1

GENERAL INTRODUCTION

1.1 Development of Cancer

The accuracy of genetic replication is essential for the survival of each individual cell and ultimately for the maintenance of the species. The DNA replication machinery is designed to ensure the stable transfer of a faithful copy of total genetic information from parental to daughter cells. The human genome, consisting of about $3 \times 10^9$ bases, has been estimated to exhibit a replicative error of only one in $10^9$ to $10^{11}$ bases (Loeb, 1991). Despite this, endogenous and environmentally induced DNA damage may lead to stable mutations that alter genomic stability leading to cell heterogeneity and clonal evolution culminating in tumour development (Nowell, 1976). The observation that neoplastic cells possess numerous genomic alterations led to the suggestion that an early step in the process of carcinogenesis is the acquisition of genomic instability. Genomic instability can be defined as “normal and abnormal tendencies of cells to undergo mutation” (Cheng and Loeb, 1997). Initiation and progression of cancer is associated with multiple genomic events, such as altered patterns of methylation, small-scale mutations or large-scale chromosomal alterations (Coleman and Tsongalis, 1995). Tumour aneuploidy is an often observed phenomena in solid tumours, both animal and human (Sandqvist et al., 1991; Bringuier et al., 1993; Greene et al., 1995; Visscher et al., 1996). It is postulated that factors present in the tumour environment are also capable of potentiating genomic
instability (Mareel et al., 1991). These factors and their contribution to tumour
progression are, as yet, poorly understood. Our laboratory was interested in further
identifying these factors and their sources by developing a model capable of detecting
mutational events within the tumour environment.

Tumours commonly contain stromal, vascular and immune elements, such as
neutrophils, tumour-associated macrophages or tumour-infiltrating lymphocytes, each of
these capable of secreting various products under normal physiological conditions or
upon stimulation. Early work by Kadhim and Rees (1984) suggested that infiltrating host
macrophages may contribute to genomic instability and tumour progression. Lymphocyte
infiltration has been demonstrated within both animal and human tumours (Whiteside et
al., 1986). In turn, progressive tumour growth may stimulate a chronic inflammatory
response (Rosin et al., 1994). Reactive oxygen species, known to be genotoxic and
potentially released into the tumour environment by infiltrating cells or by tumour cells
themselves, may also contribute to genomic instability (Weitzman and Gordon, 1990;
Nguyen et al., 1992; An and Hsie, 1993; Emerit, 1994).

1.2 Genomic Instability

Genomic instability may be manifest as karyotypic abnormalities, gene mutations
and amplifications, cellular transformations, clonal heterogeneity, and cell cycle
disruptions. Genomic instability underlies the progressive genetic changes that lead to
the expression of the neoplastic phenotype. Mutational events that cause genomic
instability can be categorized into genetic or epigenetic factors. A change in the pattern
or level of gene expression without a change in the gene function, is an epigenetic (i.e. not a genetic) event. Genetic changes resulting in DNA damage can be classified into four groups; deletions, insertions, rearrangements and point mutations. At least 4 genetic or epigenetic modifications capable of destabilizing the genome and contributing to tumour development have been identified; (i) development of a “mutator” phenotype (Loeb, 1991; Loeb 1996); (ii) alterations of genes that regulate DNA repair or DNA mitotic apparatus (Modrich, 1994; Almasan et. al., 1995); (iii) decreased stringency of fidelity during DNA synthesis (Minnick and Kunkel, 1996); (iv) decreased transcriptional regulation (Elledge, 1996). These modifications, leading to mutations, genomic instability and subsequent increase in tumour cell mutation frequency may result in tumour cell heterogeneity culminating in a cellular growth advantage and cancer development. The mechanism underlying genomic instability, observed in the endogenous or transfected genomically integrated genes, are as yet incompletely understood and will be the basis of this research. Endogenous genes with their predetermined chromosomal location are defined as those inherent within the host cell, in contrast to the exogenous genes which are introduced into the cell genome.

1.3 Endogenous Markers of Genomic Instability

Spontaneous and/or induced mutational events may induce genomic instability which may in turn produce further mutations. Certain endogenous genes within human or animal cells are useful as markers or indicators to study the effect of mutational events on genetic integrity. Eucaryctic hypoxanthine phosphoribosyltransferase (hprt), adenine
phosphoribosyltransferase (aprt), and thymidine kinase (tk), have been extensively used for studying mutations. Either hemizygous or heterozygous forms of the genes may be useful for the study of mutational events. Typical diploid somatic cells contain sex chromosomes plus paired autosomes, called homologues, where each pair encodes for maternally or paternally derived genes. Autosomal genes may exist in alternate forms or alleles. A gene locus is said to be homozygous when both alleles are identical and heterozygous when the alleles are different. In contrast, a gene present in only one copy in a diploid cell is described as hemizygous.

1.3.1 Endogenous Hemizygous Markers of Genomic Instability

The hprt gene, commonly used in mutation analysis studies, has enabled characterization of a large number of spontaneous and induced mutations. It is a large, non-essential, X-linked gene, functionally present in a single copy in both males and females (hemizygous). For these reasons, and because procedures are available for selecting cells which either lack or contain the active enzyme, hprt is a useful marker gene for monitoring intragenic mutational events (Albertini et al., 1986). Culture medium that contains the purine analogue 6-thioguanine (6-TG) or the combination of hypoxanthine, aminopterin and thymidine (HAT) selects for HPRT⁻ or HPRT⁺ cells, respectively. The HPRT enzyme is involved in the salvage pathway of purines, hypoxanthine and guanine, which are released upon degradation of nucleic acid (Kunz, 1982). Even though hprt is a non-essential gene at the cellular level, partial lack of its expression at the systemic level may lead to hyperuricemia and a form of gouty arthritis.
(incidence of 1 in 200 among males with gout), while complete deficiency results in the Lesch-Nyhan syndrome (incidence of 1 in 100,000) (Stout and Caskey, 1985; Stout and Caskey, 1988). Although useful for detecting small scale, gene mutations, endogenous hprt has limitations for detecting large scale chromosomal deletions and rearrangements. Figure I.1, adapted from De Marini et al. (1989), illustrates the possible reasons for the limitation of mutation detection by hemizygous genetic markers. An explanation for this increased sensitivity in the detection of mutations at the site of a heterozygous gene marker comes from a study by Evans et al. (1986b) which suggests that chromosomal mutational events resulting in concomitant loss of essential neighbouring genes will render the cells non-viable unless the heterozygous chromosome compensates by providing the missing essential information. Also, mutagenic mechanisms which require homologous sequences to operate, such as homologous recombination or gene conversion, could only be detected if the marker gene is on an autosome. However, it is important to note that even though the hprt is a hemizygous gene marker, it might still undergo the previously mentioned rearrangements due to the presence of pseudogenes which may become involved in these events (Nadir et al, 1996).

Using polymerase chain reaction (PCR) amplification of various gene markers on the human X-chromosome or physical mapping with Xq26 specific probes to characterize chromosomal mutants, it was suggested that maximal size deletions of about 3.5 Mb, are tolerated at the hprt locus (Fuscoe et al., 1992; Morris et al., 1993; Nelson et al., 1994; Lippert et al., 1995a). Deletion mutants exceeding this size were not recovered presumably due to loss of neighbouring essential genes rendering some chromosomal
Figure I.1 Recovery of mutants at heterozygous versus hemizygous loci.

At a functionally heterozygous loci, the recovery of intergenic mutants is more likely to result in viable mutant recovery than at a functionally hemizygous loci. In both systems, a small scale mutation within a non-essential gene will result in a viable mutant. However, a large scale mutation, encompassing adjoining essential gene(s) is more likely to be recovered in a heterozygous system, where the essential function may be supplied by the unharmed homologous chromosome. In a hemizygous system, no homologous region is available to salvage for the lack of function, thus the mutant becomes non-viable (DeMarini et al., 1989)
Functionally Heterozygous (tk \(^{+/-}\))

Functionally hemizygous (hprt)

Viable Mutant

Non-viable Mutant
mutants non-viable. However, it may be that some mutations are more complex than a simple deletion of contiguous DNA sequences (Simpson et al., 1993; Lippert et al., 1995b). Also, recent work by Jordan and Schwartz (1994) demonstrates that X-chromosome translocations are rare when compared to recombinational events occurring between autosomal chromosomes. This suggests that chromosomal segregation or nuclear architecture may affect certain types of chromosomal aberrations as demonstrated by Cremer et al. (1996). The hprt, located on the X-chromosome, may be underestimating certain groups of mutational events due to chromosomal segregation suppressing recombinational events. From mutational analysis of the endogenous hprt gene, where large intergenic losses are likely to result in non-viability (e.g. loss of essential Pgkl gene), it can be estimated that only about 10% of mutants are a consequence of large scale deletions (Lichtenauer-Kaligis et al., 1996). By contrast, the mutational spectra for the heterozygous tk gene locus can consist of as many as 70% large scale mutations (Breimer, 1988; Moore et al., 1989; Liber et al., 1989). These mutation spectra support the suggestion that certain mutational events are underestimated if the marker gene is present on the hemizygous chromosome.

1.3.2 Endogenous Heterozygous Markers of Genomic Instability

An alternative to a hemizygous gene marker would be to select for a marker on an autosome, thus potentially capturing all types of mutations (see Fig. 1.1). One inherent problem with using endogenous autosomal genes (tk and aprt) as markers of mutational events is that these genes are normally disomic (present in two copies) in their expression.
Certain mutational events, such as large scale deletions, interchromosomal homologous recombinations or gene conversions, are more easily detected if the gene of interest is an autosomal monosomic marker (present as single copy). A number of different cell lines have been established where one of the autosomal marker genes has been mutated, resulting in selection of a cell line expressing heterozygosity at that particular locus.

**Thymidine kinase** (TK) catalyses the phosphorylation of thymidine to thymidine monophosphate as part of the pyrimidine salvage pathway. TK6, a human B-lymphoblastoid cell line derived from a human male spleen, was mutated at its autosomal *tk* locus to provide a heterozygous gene marker of mutational events on chromosome 17 (Thilly et al., 1980). Gene expression is selected for in CHAT (deoxycytidine, hypoxanthine, aminopterin and thymidine). Like *hprt*, *tk* encodes for a non-essential salvage pathway enzyme involved in nucleotide metabolism which, if damaged, results in trifluorothymidine (TFT) selectable mutants. This cell line is particularly useful because it is sensitive to both gene and chromosomal mutational events (Li et al., 1992). A study by Moore at al. (1989) using Chinese hamster cells and mouse lymphoma cells, demonstrates that certain radiomimetic chemical mutagens/carcinogens, known to cause DNA double strand breaks associated with chromosome damage, induce more recoverable, albeit slower growing mutants at the *tk* locus than at the *hprt* locus, while mutagens that cause normal growing mutants due to gene damage induce a comparable number of *tk* and *hprt* mutants. Furthermore, Liber et al. (1989) support Evans' hypothesis (discussed earlier) by demonstrating a 6-fold increase in the number of mutants recovered at the *tk* gene as compared to that at the *hprt*
gene locus following exposure of TK6 cells to clastogenic agents, such as X-rays, known to induce DNA double strand breaks. This increase in number of mutants could not be explained by the gene target size theory based on the 57 kb \textit{hprt} and 12.9 kb \textit{tk}, since the target size of the genes would be in the order of 4 times in favour of the \textit{hprt} gene (Lin et al., 1983; Edwards et al., 1990). The target size theory states that the probability of a mutational hit occurring is random and dependant upon the size of the target and the extent that region can tolerate change. The increase in recovery of \textit{tk} mutants, reported by Liber et al. (1989), was only detected if the cultures were allowed to accumulate slow growing mutants, generally associated with cytogenetically visible alterations such as chromosomal rearrangements. It is assumed that a gene (or genes) neighbouring the \textit{tk} gene on human chromosome 17 is essential to be present in two copies to ensure normal cell growth. If one of the alleles is lost through a mutational event, the normal growth rate is affected. However, Li et al. (1992) demonstrated that some slow growing \textit{tk} mutants were a consequence of point mutations with a high level of concurrent mutations at a number of minisatellite loci, suggesting that a number of genes may have been affected. Thus, an alternative hypothesis is that a coincident mutation in a growth controlling or DNA repair regulating gene, not necessarily on chromosome 17, has occurred simultaneously. At present, \textit{tk} is the principal heterozygous marker gene used in mammalian mutagenesis studies.

\textbf{Adenine phosphoribosyltransferase} (APRT) is encoded by the autosomal \textit{aprt} gene, therefore each normal cell possesses two functional copies. It is a non-essential gene that catalyses the synthesis of adenosine-5’-monophosphate (AMP) from adenine
and 5-phosphoribosyl-1-pyrophosphate (PRPP). Like tk, it has been utilized as an
autosomal marker of genomic instability in selected cell lines where one of the two genes
has been lost or inactivated. A derived hemizygous strain of the Chinese hamster ovary
(CHO) cell expressing a small (2.6 kb) (De Boer et al., 1989) aprt gene and a 15 kb
deletion of the complementary allele, has been employed in many mutational analysis
studies (Grosovsky et al., 1988; De Boer and Glickman, 1992; Povirk et al., 1994; de
Jong et al., 1996). This particular cell line faces the same limitations as do the hemizygous
hprt cells for detecting large scale mutational events and thus underestimating the damage
to the chromosomes. Bradley et al. (1988) demonstrated that x-ray induced mutation
frequencies at the aprt gene could differ by as much as 100-fold depending on whether
the gene was physically hemizygous or heterozygous. Complementary observations were
made by Hakoda et al. (1990) where aprt" mutants arose in cells of aprt"" individuals at a
frequency 100-200 fold greater than that observed at the hprt locus. Harwood et al.
(1993), employed a human tumour cell line expressing heterozygous aprt genes, to
demonstrate that the high frequency of loss (5.9 x 10^-6) at the aprt was primarily due to
allele loss (multilocus chromosomal losses), followed by a 100 to 200-fold lower
frequency of base substitutions, small deletions, or duplications (approximately 10^-8).
This suggested that the loss of the remaining gene was limited to small scale mutational
events due to a newly created hemizygous, and thus less sensitive, system of mutant
detection. Similar observations were also made in the human TK6 cell line (Smith and
Grosovsky, 1993b). Since aprt is located on chromosome 16q24, it is of interest to note
that this chromosome is disproportionately gene-dense, with one of the genes encoding for

-10-
cytochrome oxidase (\textit{cox}, the terminal oxidase in mitochondrial electron transport) positioned at 16q24.1. For these reasons a chromosome 16 hemizygous marker, like \textit{aprt}, is more susceptible to viability loses as a consequence of multilocus deletions. Moreover, extensive chromosomal scale alterations have been associated with human chromosome 16 often found in tumour cells. Smith and Grosovsky (1993a) using TK6 cells, homozygous at the \textit{aprt} locus, suggested that specific sequences at 16q12 may be involved in initiating genetic instability/fragility. This characteristic of chromosome 16 would make it highly susceptible to genomic instability and possibly lethal damage. Thus, a marker gene present on this chromosome may have limitations as an indicator of mutation frequencies and may not be representative of random mutational events occurring throughout the cell.

1.4 "Fragile Sites" and Genomic Instability

The above findings pose the question of whether mutational events, spontaneous and induced, are randomly distributed over the entire genome, affecting all regions with equal frequencies. A number of studies suggest that the accumulation and expression of mutational events within a marker gene may be affected by the actual chromosomal location of that gene (Bunch et al., 1995). Under \textit{in vitro} conditions, specific \textit{fragile sites} (regions that exhibit karyotypic constrictions, gaps, or breaks under certain conditions, both spontaneous and induced) have been demonstrated in chromosomes (Sutherland and Hecht, 1985). Some of these same sites may also coincide with preferential chromosome break points \textit{in vivo}, an observation of considerable significance to cancer development.
(Yunis and Soreng, 1984; Le Beau and Rowley, 1984). A recent report by Wenger (1995) suggested that 55 to 60% of fragile site breaks induced by chemical agents correlated with known cancer breakpoints. However, statistical analysis (Mariani et al., 1995) contradicts the above suggestion by reporting no direct association between 10,492 known breakpoints with known fragile sites. Two distinct types of fragile sites have been characterized. Rare (heritable) fragile sites can be characterized as gaps or breaks, usually involving both chromatids, occurring at specific chromosomal loci when metaphase spreads are examined after treatment with inhibitors of DNA replication. This inherited fragility is expressed by production of acentric fragments, deleted chromosomes, or genetically unstable triradial chromosomes (Sutherland, 1979a, 1979b). Common (constitutive) fragile sites are characterized as frequent, if not ubiquitous, present in more than 50% of the population, and predominantly consisting of chromatid-type lesions caused by environmental factors, chemicals, radiation and/or viruses (Sutherland and Hecht, 1985). Whether radiation causes random DNA damage or site specific damage through improper activation of endogenous regulatory elements such as DNA binding proteins or alterations in specific DNA secondary structure, is still a controversial topic (Goodhead et al., 1993; Knehr et al., 1994; Bunch et al., 1995). A non-random distribution of mutagen-induced chromosome breaks was demonstrated in malignant cells treated with either radiomimetic or UV-mimetic drugs (Dave et al., 1994). In this experiment, in different patients, chromosomes 1, 6, and 9 demonstrated a disproportionately higher number of breaks with a tendency for clustering. Subtelomeric regions, interstitial telomeric sequences, and repetitive sequences may form secondary
DNA structures leading to formation of *fragile sites* associated with breakpoints in chromosomes (Bouffler et al., 1993; Day et al., 1993; Balajee et al., 1996). In the absence of any known external mutagenic agent, normal cell metabolism can induce localized spontaneous mutations resulting in chromosomal fragility.

### 1.5 Engineered Markers of Genomic Instability

To create a more suitable model for detecting all types of mutational events (both small and large scale) and at the same time verify if genomic positioning (of the marker gene) can affect the accumulation of mutations, more recent investigations have taken a different approach. Many mutational analysis studies have been conducted in cell lines where a marker gene can be, supposedly, randomly introduced to provide a heterozygous situation. The use of transfected marker genes has facilitated genomic instability research. The genes most commonly used are the *Herpes simplex* virus thymidine kinase (*tk*) and the bacterial guanine phosphoribosyltransferase (*gpt*) genes, that, upon stable integration into the eucaryotic genome, provide easily selectable markers. With the initial assumption that the integration of these foreign sequences is random (Palmiter and Brinster, 1986), this type of model would provide a means of studying the effects of the chromosomal environment on the stability and expression of the integrated genes.

#### 1.5.1 Factors Affecting Genomic Instability of Transfected Markers

Transfected marker genes, such as *tk* and *gpt*, can be considered foreign to the eucaryotic cell line and thus their transcription can be inactivated by specific mutational
events recognizing foreign sequences. Studies by Gebara et al. (1987) and Tindall and Stankowski (1989) demonstrated that most gpt transfectants of an hppt deficient human fibroblast cell line lost their transgene function at a very high frequency (1-5%). Inactivation of even the most stable transfected gene can occur at a frequency of about 4 × 10^{-3} to 1 × 10^{-3}. This observed frequency is up to 1000-fold greater than the expected spontaneous mutation frequency for an endogenous heterozygous mammalian locus such as tk (10^{-6} - 10^{-5}) (Giver and Nelson, 1995).

Hypermutability of transfected genes may be due to (I) the presence of prokaryotic genomic sequences in the transgene, (ii) site of integration which may not be completely random (as often assumed), (iii) multiplicity of integrated sequences, or (iv) presence of homologous sequences within the host cell.

Integration of prokaryotic sequences into eukaryotic host cells is one means of inducing genomic instability in host cells. Integration of foreign DNA (Adenovirus type 12, plasmid, or bacteriophage λ) into the hamster genome has been shown to exert a distinct trans effect on the methylation of cellular DNA segments located on a number of chromosomes (Heller et al., 1995). Doerfler (1991) proposed that de novo methylation of transgenes and foreign DNA can be regarded as a cellular defence mechanism against the invading DNA. This hypermethylation of the newly integrated DNA has been linked to gene silencing and inactivation of the foreign DNA, with some suggestions that de novo methylation is capable of spreading into the surrounding endogenous sequences (Zion et al., 1994; Mummaneni et al., 1995). Hypermethylated regions appear to undergo higher chromatin condensation which, in turn, may be associated with decreased genomic
transcription (Antequera et al., 1989). In addition, chromatin protein histone H1 appears to bind preferentially to methylated inactive DNA and in this manner more effectively inhibits the initiation of RNA synthesis (Levine et al., 1993). Through these means of local transcriptional inactivation, each specific site of integration could potentially result in a unique epigenetic expression of the transfected cell (Doerfler, 1995). It is now well established that methylation of CpG islands is species-, cell type- and DNA segment-specific, and is dependent upon the stage of cellular development which, if disrupted, could result in differential gene expression. A recent publication (Radtke et al., 1996), supports the theory of differential transcriptional inactivation by methylation sensitive transcription factors.

Aside from changing methylation patterns, integrated foreign eucaryotic sequences have also been associated with morphological changes in DNA structure (McManus et al., 1994). They transfected yeast DNA into mouse DNA which results in cytogenetically differentiated regions, closely resembling fragile sites, with lower compaction and decreased propidium iodide staining irrespective of the level of DNA methylation. However, it has been suggested that mammalian metaphase chromosomes can undergo a degree of compaction up to 5 times that observed in yeast cells. This compaction does not appear to correlate with nucleosome structure since yeast and mouse DNA are packaged similarly. Therefore, it is possible that both prokaryotic and eucaryotic foreign sequences could lead to genomic instability upon transfection into mammalian cells due to structural differences in the DNA caused by 2nd or 3rd order of compaction (helical arrangement of nucleosomes into solenoids and folding of solenoids into loops).
To reflect the inherent or endogenous situation for detecting mutational events, it is preferable that a test system should contain no foreign DNA. Ideally, the mutational mechanisms functioning at the site of integration should be representative of those occurring in the rest of the genome, assuming that the site of integration was random. Until now, no genomic stability studies have been conducted on transfected genomic sequences that were inherent to the host cell and did not contain any foreign sequences.

The site of integration of the transfected sequences is also a factor that may contribute to the observed hypermutability and genomic instability. Aside from the obvious effect where a transgene might integrate into an endogenous coding sequence, resulting in an insertional mutation, other not so obvious regions could also contribute to genomic instability. Work by Liehtenausser-Kaligis et al. (1993), utilizing the TK6 cell line transfected with hamster hprt cDNA and the neo genes transcribed from the retroviral long terminal repeat (LTR) promoter, suggests that spontaneous mutagenesis in a gene is dependent on its position in the genome. HPRT⁺ mutations differed by up to 60-fold in the five retroviral integrants studied, ranging from $5.9 \times 10^{-6}$ to $3.5 \times 10^{-4}$ mutations per cell per generation (Lichtenauer-Kaligis et al., 1996). It was demonstrated that both mutation rate and mutation spectra were different for each of the transfectants. Even though the integration sites were assumed to be random, it is important to consider that they may be influenced by the presence of fragile sites, homologous endogenous sequences or the presence of pseudogenes. Human hprt gene has four autosomal pseudogenes that have been mapped to chromosomes 3, 5, and 11 (Patel et al., 1986). Thus, it is possible that site directed integration through reciprocal exchange is distorting
the randomness of the event (Van Dijk et al., 1996). Hoeben et al. (1991) also demonstrated that inactivation of a transfected beta-galactosidase and a neo gene construct, flanked with Moloney murine leukemia LTR sequences, was dependent on their chromosome location. Furthermore, inactivation of the gene was often associated with methylation of the viral sequences. Homologous recombination is not likely to play an important role in the integration of viral sequences. The above cited experiments emphasize the site of integration as an important factor in maintenance of gene expression.

Aside from the possibility that homologous recombination may bias the site of integration (see page 21 for discussion), other factors may also affect the sites of integration. An interesting correlation between subtelomeric regions containing high density of CpG clusters and high density of genes (Saccone et al., 1992) with regions of increased recombinational activity, possibly fragile sites (Glover and Stein, 1987), may facilitate integration of transfected sequences into these specific sites. Studies by Kato et al. (1986) suggest that transfected DNA may preferentially integrate into repetitive sequence DNA, which in turn may affect insert stability or gene expression, and thus further contribute to increased mutation rates. As reviewed by Leach (1994), and demonstrated by Usdin and Woodford (1995), these regions could be more susceptible to double strand breaks, deletions or amplifications. If these double strand breaks occur in repetitive sequences surrounded by gene rich regions, the resulting genomic instability may be more pronounced. The generation of a DNA double strand break is a potentially lethal event, if not repaired prior to progression through the cell cycle, because essential
genetic information on the acentric fragment could be lost (Iliakis, 1988; Ortiz et al., 1995). The double strand break induces homologous inter- or intrachromosomal, or nonhomologous recombination repair mechanisms in an attempt to salvage the cell (Sweezy and Fishel, 1994). Thus, subtelomeric regions, interstitial telomeric sequences, repetitive sequences, or other common or rare fragile sites that are associated with double strand break formation could be regions where recombination occurs at a higher rate than would be observed in the rest of the genome (Hastie and Allshire, 1989; Maercker and Lipps, 1993; Balajee et al., 1994; Tóth and Jurka, 1994) and reviewed by (Laird et al., 1987; Sutherland and Richards, 1995). Insertion of sticky-ended transgenes (with or without terminal protective telomeric sequences) could occur during the DNA double strand break repair processes, possibly resulting in integration of tandem arrays.

Some evidence also supports the suggestion that the initial event in the process of gene amplification may be a double strand break in DNA leading to chromosomal breakage-fusion-bridge cycle (McClintock, 1951), commonly resulting in head-to-head organization and frequent deletions (Jorgensen et al., 1987; Windle et al., 1991). Evidence that gene amplification does occur within regions where double strand breaks are known to occur, such as, telomeric or interstitial telomeric sequences is provided by Bertoni et al. (1994) and Amann et al. (1996). This amplification could be stable, or lead to structural DNA breakage with subsequent genomic instability and genomic losses. The association between fragile sites and viral sequence integration is now supported by Wilke et al. (1996) who provides the first evidence that chromosomal common fragile sites can accommodate spontaneous HPV16 (Human papilloma virus) viral integration.
A previous study by Rassool et al. (1991) suggested this possibility by demonstrating, under *in-vitro* conditions, a preferential integration (4/13) of marker DNA (pSV2neo) into aphidicolin induced *fragile sites* on chromosome 3. Their protocol uses aphidicolin to inhibit DNA polymerase α and δ, delaying DNA replication which leads to production of *fragile sites* (Glover et al., 1984). They next demonstrated, by fluorescence *in situ* hybridization (FISH), that these same sites of integration underwent increased genetic instability, particularly chromosome rearrangements, when compared to non-transfected sites (Rassool et al., 1992). Two characteristics of *fragile sites* that may possibly facilitate the incorporation of genomic sequences are their tendency to occur in active gene rich regions (Hecht, 1988) and their apparent association with late replicating regions, possibly reflecting a delay due to DNA methylation (Laird et al., 1987).

**Multiplicity of integrated sequences** may be dependent on the method by which the new DNA is introduced into the cell and may also govern the final stability of transfected genes. Electroporation is usually associated with single-copy, single-site gene integrations but coincident insertions or subsequent amplification of inserted sequences may lead to multiplicity of the gene. Transgenic experiments conducted in plants by Jorgensen et al. (1987) suggest that only 10% of the transfectants will contain a single copy of the new DNA. They demonstrated that the inverted (head-to-head or tail-to-tail) repeats predominated, but both inverted and direct repeats (head-to-tail) may participate in a single array. This orientational bias would argue against a ligation mechanism occurring between fragments prior to integration into plant genome. A possible explanation would be that the genomic sequences are amplified post-integration
possibly through a breakage-fusion-bridge mechanism (see page 18).

Duplicated genomic sequences (tk), integrated at different sites in the viral genome (Vaccinia virus) are subject to extensive recombination, both translocations and inversions (Kriajevskas et al., 1994). Tandem insertion of DNA sequences into Neurospora crassa, could also activate rec-2-mediated premeiotic excision of these invading genes and thus provide an independent means for eliminating duplicated sequences (Bowring and Catchmarked, 1993). Recent studies done on Neurospora crassa and Ascobolus immersus suggest that inactivation of duplicated gene-sized sequences, not necessarily linked, may activate a repeat induced point (RIP) mutation, or a methylation induced (MIP) mechanism in premeiotic cells (Rhounim et al., 1992; Singer and Selker, 1995). These mechanisms may be the means by which a cell protects itself against invading or proliferating genes. The suggestion is that RIP activation by DNA-DNA interaction between homologous sequences causes de novo methylation, followed by point mutations (G:C to A:T) which neutralize the duplications (Singer et al., 1995). MIP, also dependent upon DNA-DNA homology, is an inactivating nonmutagenic event in fungal cells involving a reversible methylation process. This type of reversible inactivation was also demonstrated in a transgenic tobacco cell line which was subsequently transfected with a construct that had partial homology to the first transgene (Matzke et al., 1989). Unidirectional inactivation of the first transgene occurred in 15% of the recipients of the secondary transfection, irrespective of the site of integration. Whether the above mentioned mechanisms are available and functional in somatic mammalian cells remains to be investigated (Kricker et al., 1992). If there is a
comparable mechanism in mammalian cells, it would be interesting to determine its ability to distinguish and maintain the fidelity of endogenous repetitive sequences such as rDNA and its capacity to eliminate invading DNA. The suggestion that homology greater than 85% is essential for homologous recombination would explain why Alu repeats or repetitive gene-sized fragments, which express decreased homology, could be faithfully maintained (Waldman and Liskay, 1988; Kricker et al., 1992). Since the efficiency of recombination appears to require a minimum of 300 bp of uninterrupted homology, a mammalian cell can, by breaking up genes into smaller exon sequences spanned by larger intronic sequences, prevent recombination between genes and their pseudogenes (Liskay et al., 1987).

Homology between the host cell genome and transfecting sequences may also affect hypermutability and randomness of integration. As implied earlier (page 17) endogenous homologous sequences could act as templates for recombinational events thus biasing the site of integration to these regions of homology. Aside from creating a bias for the site of integration, the presence of homologous sequences could also affect the stability of the integrated gene. Initial gene integration may be facilitated by homologous sequences; however, the maintenance of the transgene may be jeopardized if the homology is too great.

Many factors capable of affecting gene transfection experiments are just now being elucidated. Further understanding of genomic instability of transfected sequences may enlighten the efficacy and optimize potentials of gene therapy.
1.6  THESIS OUTLINE

This thesis investigates genomic instability in two separate models. A mouse fibrosarcoma MC1A-C1 cell line expressing a heterozygous hprt gene was used to examine genomic instability at an endogenous genetic marker. In developing this mouse cell model, the need to investigate the relationship between the chromosomal site of the gene marker and the sensitivity of the cells to detect mutational events became apparent. Knowing this relationship might assist in identifying factors that modulate the susceptibility of a specific genetic marker to mutational events, and why the susceptibility of the gene might vary with, or depend upon, its location within the chromosome. For this purpose, in the second part of this thesis, human lymphoblastoid TK6 cells transfected with a marker gene, presumably integrating in different chromosomal locations, were used to examine genomic instability at transfected genetic markers.

1.6.1 Genomic Instability of Endogenous hprt Gene in a Mouse Fibrosarcoma

The objective of the first section of this thesis was to develop a transplantable mouse fibrosarcoma cell line that could detect, with improved sensitivity, mutational events that may be induced by factors present in a tumour environment. We designed a model system to monitor in vivo mutations at a heterozygous hprt genetic marker, based on the hypothesis that factors in the tumour environment, such as reactive oxygen species, could actively contribute to genomic instability (Birnboim, 1983a; Emerit and Cerutti, 1983; Heppner et al., 1984). This model has the potential to identify and study the effect of various endogenous and exogenous cellular factors on tumour progression in vivo and

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to compare these results to spontaneous or induced mutational events in vitro. In response to the still debated suggestion that nitric oxide could be mutagenic (Felley-Bosco et al., 1995), our laboratory set about to apply this sensitive model to determine if mutational events could be induced within the tumour environment by the action of infiltrating immune response cells known to be capable of releasing nitric oxide. Other experiments are also underway in which these model cells are being used to study the effects of external factors such as ionizing (Co\(^{60}\)) or non-ionizing radiation (60 Hz Electromagnetic fields) on the genome.

1.6.2 Genomic Instability of Transfected \textit{hprt} Gene in a Human TK6 Lymphoblastoma Cell

The second part of this thesis examines the genomic instability of a transfected \textit{hprt} gene in a human tumour cell line. The study uses a number of methods and biological endpoints to examine genomic instability, including chromosome aberrations, and fluorescence \textit{in situ} hybridization, to potentially localize the transfected fragment. Because of the inherent cytogenetic advantages and the relevance of genomic instability and gene therapy studies to human cancers, a human TK6 cell line was selected for these studies. A number of clones expressing a transfected human \textit{hprt} minigene were studied for spontaneous or induced instability of gene expression. The \textit{hprt} minigene, a functional condensed version of the endogenous \textit{hprt} gene, used in this study is described in Figure II.1. This study follows the fate of a transfected human gene that does not contain any viral, bacterial, or plasmid sequences. The presented data and future
characterizations of these clones could lead to better understanding and planning of gene transfection experiments which are of consequence in gene therapy and transgenic animal studies.
CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1 DESCRIPTION AND ORIGIN OF THE CELL LINES

MC1A was a mouse fibrosarcoma cell line adapted to in vitro growth and genetically manipulated to allow selection of a number of mutant clones expressing hprt as a heterozygous marker. More detailed description of clone derivations and summary of derived clones is presented in section 3.2 and Appendix I.

SP2/0 mouse myeloma cell line (ATCC) is known to be deficient at the endogenous hprt gene locus. Further description of hprt transfectants is presented in section 3.3 and Appendix I.

A human neuroblastoma cell line (IMR-32), a human colon cancer cell line (LS-174T), and a normal human fibroblast were potential candidates for study of genomic instability of transfected hprt gene. TK6, a human lymphoblastoid cell line derived from a spleen of a male patient, was selected for mutation and transfection with the hprt marker as indicator of genomic instability. Detailed descriptions of these cell lines and their derived clones are presented in section 4.2.

2.2 CELL CULTURE CONDITIONS

2.2.1 Non-Selective Conditions

Mouse fibrosarcoma MC1A-C1 cells, SP2/0 myeloma cells and their derivatives,
human fibroblasts and LS-174T colon cancer cells were grown in Dulbecco’s modified Eagle medium (DMEM), while human TK6 cells and its derivatives were grown in RPMI 1640 medium. Both media were supplemented with 10% fetal calf serum (FCS) (Gibco BRL, Burlington, Ontario, Canada) and cells were cultured in 5% carbon dioxide / 95% air at 37°C. Methylcellulose-RPMI 1640 medium was used for TK6 cells to allow colony formation by these suspension cells (Buick et al., 1979). This medium was prepared by adding RPMI 1640 medium, FCS and L-glutamine to a stock solution of methylcellulose in Iscove’s MDM (Stemcell Technologies Inc. Vancouver, B.C. Canada) to yield a final concentration of 0.8% methylcellulose in RPMI 1640/Iscove’s MDM. Human IMR-32 neuroblastoma cells required 30% FCS for optimal growth in DMEM. All adherent cells were cultured to subconfluency while suspension cells were maintained at densities ranging from 1 to 10 x 10^5 cells ml^-1. All cultures were maintained in the absence of antibiotics and tested periodically for presence of Mycoplasma by fluorescent Hoechst 33258 (Sigma Chemical Co., St. Louis, MO. USA) stain method (Chen, 1977).

2.2.2 Selective Conditions

HAT medium, used for selecting HPRT^- cells, was non-selective medium supplemented with hypoxanthine, aminopterin and thymidine at concentrations of 1x10^-4 M, 4x10^-7 M, and 1.5 x 10^-5 M, respectively. CHAT medium, selecting for both hprt and tk expression, was HAT medium containing 10^-5 M deoxycytidine. Cells lacking functional HPRT were selected in 6-TG medium (non-selective medium supplemented with 5x10^-5 M 6-thioguanine); cells lacking functional TK were selected in TFT medium.
(non-selective medium supplemented with trifluorothymine deoxyriboside, 1.7x10^{-4} M). All the above chemicals were purchased from Sigma Chemical Co., St. Louis, MO. USA. During the selection protocol of adherent cells, culture medium was replaced twice weekly with fresh non-selective medium containing the appropriate drug(s). SP2/0 and TK6 suspension cells in 96 well culture dishes were fed with 50 μl of fresh selective medium, at intervals of 5 days. 6TG^{R} and HAT^{R} colonies were scored at 14 days and TFT^{R} colonies were scored at 14 and 21 days to allow for the growth of slow growing mutants. The transfer of cells from either HAT or CHAT to non-selective conditions required a 2 day adaptation in HT or CHT respectively (at the concentrations of hypoxanthine, thymidine and deoxycytidine stated above) to minimize the lethal effects of any residual aminopterin. G418 medium, used for selecting neo gene expression, was non-selective medium supplemented with 500 μg ml^{-1} G418 (Gibco BRL, New York, NY. USA.)

2.2.3 Cell Counts and In Vitro Growth Rate Determination

The number of viable cells was determined by a dilution of cell suspensions with an equal volume of 0.4% w/v trypan blue in BSS (Hanks’ balanced salt solution, Gibco BRL, Burlington, Canada). After allowing 2 min for dye uptake by non-viable cells, counts of clear versus stained cells was carried out using a haemocytometer counting chamber. For each cell culture, appropriate dilutions were made to allow scoring of 30 to 300 cells per field, and in each case two separate fields were scored. The in vitro growth rates of all cell lines, and some derived clones, were established in a similar manner. A
known number of cells (1 x 10⁴), were plated per 6 cm dish and the increase in cell
number (average of at least three plates) was monitored daily for 7 days using a
haemocytometer or a Coulter Counter. Cell doubling times were estimated from the
logarithmic phase of the growth curve.

2.2.4 Scoring of Resistant Colonies

MC1A-C1 or derived clones were monitored for spontaneous or induced drug-
resistance by plating 1x10⁵ cells per 10 cm tissue culture dish. Following 14 days of
selection, or sufficient time to allow colony formation, dishes were washed in PBS
(Dulbecco’s calcium-magnesium-free phosphate buffered saline, Gibco BRL, Burlington,
Canada), colonies were fixed in methanol for 20 min, rinsed with PBS and stained with
Wright’s stain for 5 min. Excess stain was removed by gentle washing with running
water. Up to about 300 colonies (>50 cells per colony) per 10 cm dish could be readily
scored under 40 x magnification. All results are expressed as mutants per 1 x 10⁶
clonable cells, i.e. corrected for experimentally determined plating efficiency (the number
of colonies divided by the number of cells seeded).

Suspension cells (TK6, SP2/0 and their derived clones) were also monitored for
spontaneous and induced drug-resistance. Two different culture techniques were
employed. The original technique, applied to both TK6 and SP2/0 cells and mutants,
consisted of plating 1 x 10⁵ cells in 10 ml of RPMI 1640 or DMEM respectively, per 96
multiwell flat bottom tissue culture plate (approximately 1 x 10³ cells/well). In later work
with TK6 cells, 1 x 10⁵ cells/well of a 6-well flat bottom tissue culture plate were
challenged in selective methylcellulose-RPMI 1640. Plating efficiencies were established for each specific culture condition by determining cell viability under non-selective conditions. All wells were scored for growth under 40 x magnification at 14 and 21 days. Methylcellulose-RPMI culture plates were handled gently to minimize disturbance of colony formation in the semisolid medium. Results were corrected for plating efficiency and reported as mutants per 1 x 10⁶ viable cells.

2.2.5 Determination of In Vivo Mutation Frequencies

Female mice, C57BL/6, 8-10 weeks of age (Charles River Laboratories, Raleigh, NC, USA) were acclimatized for 7 days prior to use. During this time animals were fed standard chow and water, ad libitum, and maintained in a 12 h light/dark cycle. At the initiation of each experiment, 1 x 10⁶ mouse fibrosarcoma cells in 0.1 ml PBS were injected subcutaneously into the left flank of each animal. When tumours reached approximately 1 cm in size (15-21 days after injection), animals were euthanised by CO₂ asphyxiation followed by cervical dislocation. Tumours were excised under aseptic conditions and cell suspensions were prepared by mechanical disruption in 10 ml of non-selective DMEM. Cells suspensions were incubated for 2-4 days in non-selective medium to allow cell attachment of viable cells and to facilitate removal of debris. Viable cells (1 x 10⁵ per 10 cm dish) were replated in 6-TG selective medium for scoring of 6-TG^R mutants. Cells were also plated in non-selective medium for determination of plating efficiency and neo infected MC-TGS17-51 cells (and derived clones, section 2.3.1) were cultured in G418 medium to establish percent of host cell contamination. The

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reported number of 6-TG<sup>R</sup> mutants was corrected for experimentally established plating efficiency and neo resistance. All animal procedures were carried out in accordance with guidelines of the Canadian Council on Animal Care and the Animals for Research Act RSO-190.

2.2.6 Subcloning Procedures

Both mouse fibrosarcoma and human TK6 cells were periodically subcloned. A population of cells was diluted to a concentration of 30 cells per 10 ml non-selective medium and seeded onto a 96 well tissue culture plate (100μl/well). Plates were incubated, fed as needed, and monitored for growth at the end of 14 days. From each plate, a single well demonstrating colony formation was selected as a clone. In the case of suspension cells, this cloning procedure was repeated twice to ensure recovery of a pure clone.

2.3 DNA TRANSFECTIONS

2.3.1 Infection of MC-TGS17-51 with neo Gene to Confer G418 Resistance

The neo gene was derived from a DOEJS retroviral vector (Bennett et al., 1994). This selectable marker was useful for in vivo mutation studies, allowing selection of MC-TGS17-51 (expressing the neo gene) cells from a background of non-infected, geneticin (G418) (Sigma Chemical Co., St. Louis, MO. USA) sensitive host cells. Cells were plated (1x10<sup>5</sup> cells per 10 cm dish) and exposed to 5 ml of media infected with a transfection cocktail consisting of neo retrovirus (kindly provided by Dr. S. Bennett) in
the presence of 4μg ml⁻¹ polybrene, a polycation (Coelen et al., 1983). Following an 18 h infection period, cells were washed and incubated with fresh medium prior to challenge with G418 at a final concentration of 0.5 mg ml⁻¹, 48 hours after exposure to the infecting virus.

Following tumour excision, transfected MC-TGS17-51 cells infected with the neo transgene could be distinguished from contaminating mouse host cells (e.g., fibroblasts). Resistant cells were cultured under selective conditions for 14 days prior to scoring. A ratio of the number of G418 resistant colonies to the total number of colonies obtained from a parallel experiment assessing total number of viable cells in non-selective medium, was used to correct for contamination by host cells in the calculation of in vivo mutation frequencies.

### 2.3.2 DNA Transfection

Human hprt minigene (5μg), cut from the pHPT37-D2 plasmid vector (Chang et al., 1986; Patel et al., 1986) (Fig. II.1) and dissolved in 200 μl H₂O, was filter sterilized, checked for recovery on 0.8% agarose gel and used in transfection experiments.

Transfection of MC-TGR17 6-TG¹ cells was attempted by electroportating 8x10⁶ cells in 0.8 ml of 0°C HEPES buffered saline. Cells, on ice, were subjected to 1180 μfarads (μF) at 300 volts setting on a Gibco BRL cell porator (Life Technologies, MD, USA). Cell viability was assessed at 40% by the trypan blue exclusion assay using a haemocytometer, as described earlier. After a 15 min incubation on ice, 1 x 10⁶ cells were seeded per 96 well tissue culture plates. Each day, starting on day 2 and continuing
Figure II.1  pHPT37-D2 plasmid vector with the human *hprt* minigene.

The minigene consisting of its own endogenous promoter, 9 exons interspersed with partial introns 7 and 8, and a human poly-A signal was incorporated into the pUC 8 plasmid. The complete 3.0 kb minigene was excised with *EcoRI*, purified and utilized in transfection experiments.
pHPT37-D2

0.7 kb

HindIII
BamHI
EcoRI

XhoI

HindIII
AvaII

-234

Exon 1-7

ATG

amp

pUC 8 (2.7 kb)

5.0 kb

Exon 9

8

TAA

AvaII

AvaII

EcoRI
until day 8, a fresh plate of cells growing in non-selective medium was challenged with HAT. Plates were incubated for 14 days in selective media and resistant colonies were recovered. In total 54 HAT\textsuperscript{R} colonies were selected and re-cloned.

TK6 6-TG\textsuperscript{R} cells cultured in RPMI 1640 were preincubated in RPMI 1640 (without FCS) supplemented with 10 mM dextrose (Difco Laboratories, Detroit, Michigan, USA) and 0.1 mM dithiothreitol (Bio-Rad Laboratories, Mississauga, Ontario, Canada). Linear 3.0 kb human \textit{hprt} minigene cut from the pHPT37-D2 plasmid vector or intact plasmid (section 2.3.2), was dissolved in H\textsubscript{2}O, filter-sterilized and the DNA concentration determined. A volume of 25 \textmu{l} (10 \mu g DNA) was added to an electroporation volume of 300 \textmu{l} of cell suspension (1.3x10\textsuperscript{7} cells per ml) in a Bio-Rad Cuvette (0.4 cm gap). The gene pulser cell porator (Bio-Rad, Life Sciences, Hercules, CA, USA), was set at 960 \mu F and a voltage of 320V to deliver a 46 msec pulse at 0.8 kV/cm at room temperature. Two parallel experiments were conducted; the first, using the intact plasmid was to establish the functionality of the transfected minigene, and the second, using the linearized fragments was to select permanent transfectants containing only eukaryotic DNA. Cell viability, by trypan blue exclusion, was estimated for each experiment.

Transient expression of \textit{hprt} minigene in TK6 cells was determined after electroporation by culturing the cells in non-selective medium for 48 hours and then selecting for gene expression in HAT medium. After 3 days of selection in HAT medium, triplicate plates were counted and survival was estimated by back extrapolation. On day 2, the calculated efficiency of transfection, total of transient plus permanent, was
estimated at 2 to 3%. Permanent transfection was estimated at 0.1% by scoring the number of surviving colonies in long term culture in selective HAT-methylcellulose-RPMI 1640 medium.

For isolation of permanently transfected TK6 clones, immediately following electroporation, cells were subcultured into two 96 well tissue culture plates (5x10^5 cells/plate) in non-selective medium. Following a 4 day expression period for hprt, cells were challenged with HAT and then cultured for up to 21 days. Using the calculated values of 26% survival in this experiment, and 0.1% estimate of permanent transfection, each plate was expected to recover 65 mutant clones, assuming the average plating efficiency of 50%. Wells demonstrating growth were re-cloned as described in section 2.2.6. In total, 101 clones were isolated of which 9 were randomly selected for further studies and the remaining 92 frozen for future experiments. Permanent transfection for this experiment was estimated at about 0.08%.

2.4 MUTAGENESIS

To eliminate the background of HPRT− or TK− mutants, cultured mouse fibrosarcoma and human TK6 cells were treated with HAT or CHAT for 7 days prior to each experiment. Because residual aminopterin, once thymidine and hypoxanthine were removed, was toxic to the cells (data not shown), cultures were maintained in either HT or CHT for a 2 day period prior to transfer from selective to non-selective medium and treatment.
2.4.1 Cell Survival / Kill Curves

Cell survival was established after each treatment for mutation induction. A known number of cells from treated and control cultures were plated into culture medium for up to 21 days to allow colony formation. Adherent cells (mouse fibrosarcoma) were plated directly onto tissue culture dishes with appropriate non-selective liquid medium, while suspension (human TK6) cell viability was established in methylcellulose RPMI 1640 non-selective medium. Colony formation was scored as previously described (see section 2.2.4). Cell survival was expressed as a percentage (ratio of the number of viable colonies after treatment to the number of viable control colonies (no treatment) times 100).

2.4.2 Cell Irradiations.

Mouse fibrosarcoma or human TK6 cells, in non-selective medium, were irradiated with specified doses of Co$^{60}$ γ-rays (dose rate 1.8-1.4 Gy min$^{-1}$; Theratron 780, Atomic Energy of Canada). Immediately following treatment, cells were assessed for survival by either the trypan blue exclusion assay or a clonogenic assay. Following a 7 or 8 day expression time, with subculturing as needed, 1x10$^5$ viable cells (as determined by trypan blue exclusion assay) were replated and challenged with 6-TG or TFT medium for determination of mutation frequencies ($hp$rt and $tk$, respectively). Unirradiated (control) cells were similarly treated, without irradiation.
2.4.3 Bleomycin Treatment For Selection of TK6 Mutants

Fresh complex of aqueous bleomycin-iron (III) citrate was prepared at room temperature by mixing equal volumes of 2 mM bleomycin sulfate (Blenoxane, Bristol Laboratories of Canada) and a solution of iron (III) citrate (25 mM citric acid, 10 mM FeCl₃) and allowing 10 min for the complex to form prior to use. Cells were treated for 1 h at 37°C in complete medium at final concentrations of 1, 3, 10, 30, and 100 μM bleomycin. Following treatment, the cells were washed 2 x with PBS and subcultured in fresh medium. Survival assays were established immediately after treatment and mutation frequency assays following a 7 day incubation to allow full expression of mutant phenotypes. During this time cells were subcultured to fresh medium, as required, based upon the cell densities. Control cells were treated in a similar manner without the exposure to the bleomycin complex.

2.4.4 5-Azacytidine Treatment

A number of 6TG⁰ clones (MC-TGR17, and revertants of MC-TGS17-20,-50 and -51) grown for 2 days in non-selective medium were incubated for 72 h in the presence of 0, 1.0, 3.0, or 6.0 μM 5-azacytidine (Sigma, St. Louis, MO. USA). Plating efficiency and mutation or reversion frequency assays were carried out by plating a known number of cells in non-selective medium or in HAT-medium respectively (sections 2.2.1 and 2.2.2).

Survival and growth curves for TK6 cells in 5-azacytidine was established by performing plating efficiency assays and trypan blue exclusion assays over a four day period in the presence of different concentrations of the drug (0, 0.1, 0.5, 1.0, 3.0, and 6.0
For testing reversion frequencies, pooled samples of 6-TG<sup>+</sup> clones were cultured in non-selective medium for 2 days prior to treatment with 0, 1.0 or 3.0 μM 5-azacytidine. At the end of a 63 hour incubation period, cells were cultured for plating efficiency in non-selective medium and mutation or reversion frequency in HAT-medium.

2.4.5 Caffeine Treatment of TK6 Cells

Tissue culture flasks were set up in triplicate with 1x10<sup>5</sup> cells ml<sup>-1</sup> in 10 ml of RPMI 1640 medium and treated with caffeine (Sigma, St. Louis, MO. USA) at 0, 0.1, 0.5, 1.0 and 2.0 mM for 1 h at 37°C in 5% CO<sub>2</sub>. Cells were transported on ice and irradiated at a dose of 130 rads (as described earlier). Control cells were similarly treated but without exposure to radiation. Flasks were maintained on ice for 70 min following irradiation, at which point a fraction of each culture was subjected to a cell survival assay (described in section 2.4.1). The remaining cells were maintained in non-selective medium at 10<sup>5</sup> - 10<sup>6</sup> cells ml<sup>-1</sup> until tested for mutations at 8 days after irradiation (described in section 2.2.4).

2.5 MOLECULAR BIOLOGY

2.5.1 DNA Extraction

All DNA extractions were prepared according to an established protocol (Birnboim, 1992) with some adaptations. Each suspension of approximately 10<sup>7</sup> cells (mouse fibrosarcoma or TK6) was washed in PBS, and then solubilized by gentle shaking
in 3 ml of DES (1 M LiCl, 1 M urea, 5 mM CDTA, 50 mM tris pH 8.0, and 0.2% SDS). Proteinase K was added to a final concentration of 100 μg ml⁻¹ and then the lysate was incubated at 45°C for up to 14 h. 0.55 volume of isopropanol was added and the sample was gently mixed until a clot of DNA formed. The supernatant was removed and the pellet was redissolved in 300 μl CT (10 mM Tris-HCl, pH 7.5, 1 mM CDTA) containing 50 μg ml⁻¹ boiled pancreatic RNase. The DNA pellet was incubated at 37°C until solubilized and then an equal volume of DES (300 μl) was added prior to extraction with 600 μl phenol/CHCl₃ (1 g phenol ml⁻¹ CHCl₃). This extraction was repeated three times. Each extraction consisted of 30 min wrist-action shaking followed by a 2 min microfuge centrifugation. The DNA was ethanol precipitated, washed with ethanol and dried before solubilization in either CT or sterile H₂O. DNA concentration was measured by absorbance at 263 or 260 nm (CT or H₂O solubilized samples respectively). RNA contamination was estimated by determining the fraction of total nucleic acid that was rendered acid soluble after RNase digestion.

An alternate method of DNA extraction was also performed by using the QIamp DNA extraction kit (Qiagen Inc., Chatsworth, CA, USA), as per company instructions. All these samples had significant RNA contamination.

2.5.2 The Recovery of DNA Fragments From Agarose - Glass Powder Method

An agarose fragment containing the DNA of interest was cut out and recovered using a laboratory procedure similar to the Geneclean kit. The extracted DNA, in CT, was recovered and assessed for concentration by absorbance at 263 nm.
2.5.3 Plasmid DNA Extractions - Glass Powder Method

The modified method of Birnboim (1983b) was used to extract the plasmid DNA.

2.5.4 RNA Extraction

A revised method of RNA extraction, as described by Birnboim (1988 and 1992), was used in this protocol. Briefly, about $10^7$ cells were washed and resuspended in 250 $\mu$l cold PBS. After adding 3 ml of RES-1 buffer (0.5 M LiCl, 1.0 M urea, 1.0% SDS, 20 mM sodium citrate and 5.0 mM CDTA, final pH= 6.8), the DNA was sonicated for 15 s, proteinase K (1 mg ml$^{-1}$) was added to give a final concentration of 100 $\mu$g ml$^{-1}$, the mixture was incubated at 50°C for 30 min and then chilled on ice for 2 min. RNA was precipitated from the mixture by adding 2 volumes of cold ethanol and 200$\mu$l of 2 N NaOAc (sodium acetate). The pellet was dissolved in 0.9 ml RES-1 and again digested with proteinase K. RNA was extracted from this mixture with 100 $\mu$l phenol/CHCl$_3$ (repeated twice) and the extracts were combined prior to a subsequent chloroform extraction. LiCl precipitation was carried out for more than 2 h at 0°C by adding 7.5 $\mu$l of 2.0 M acetic acid and 1 ml LiCl/ethanol (3 volumes of 5 M LiCl + 2 volumes of 95% ethanol). The supernatant was discarded and the pellet resuspended in 0.4 ml CCS (1 mM sodium citrate, 1 mM CDTA, 0.1% SDS, pH 6.8) to which 35 $\mu$l of 2 M NaOAc and 1.0 ml ethanol was added. The RNA was again precipitated at -70°C for 5 min, and the pellet washed with 0.1 ml CCS. This precipitation step was repeated, and the final precipitate was dissolved in 50 $\mu$l of sterile H$_2$O and stored at -100°C until needed. RNA concentration was estimated by absorbance at 260 nm.
In extracting RNA for the purposes of slot-blot analyses, the modified protocol of Bennett et al. (1994) was used. Briefly, eleven neo infected clones of MC-TGS17-51, grown to approximately $10^5$ cells per well in 24 well plates with G418 selective medium, were washed in BSS. RNA was extracted at 50°C in 250 μl RES (0.5 M LiCl, 1.0 M urea, 0.25% SDS, 20 mM sodium citrate, 5 mM CDTA, pH 6.8) containing 50 μg ml\(^{-1}\) proteinase K. Samples were incubated, with shaking, at 50°C for 2 h, sonicated for 5 s and then sequentially extracted with 50 μl phenol/CHCl\(_3\) and 100 μl chloroform. Residual chloroform was removed by evaporation prior to sample application to the slot-blot apparatus. In parallel, 12 control colonies, not exposed to the retroviral vector were treated in a similar manner to extract RNA. All samples were assessed in duplicate.

2.5.5 Probe Preparations

The probes used for Southern analyses were restriction fragments cut from plasmids. Plasmid pHPT37-D2, kindly donated by Dr. P. Patel, was used to generate different probe fragments (see Fig. II.2a). The Xhol/HindIII restriction fragment of pHPT37-D2 plasmid was ligated into Xhol/HindIII cut Bluescript KS + phagemid (Stratagene, La Jolla, CA, USA) to provide an alternate probe (Fig. II.2b).

2.5.6 Southern Analysis

For Southern blot analysis, 11μg of high-molecular-weight genomic DNA was digested to completion with 3-5 fold excess of restriction enzymes, as indicated in the legends to Figures IV.12, 14 and 26 (Boehringer Mannheim, Laval, Que., Canada). One
Figure II.2a  \textit{hprt} minigene probes for Southern analysis excised from pHPT37-D2 plasmid.

(1) The entire 3 kb \textit{EcoRI} fragment of the \textit{hprt} minigene random primed. (2) Random primed 2.62 kb \textit{XhoI-EcoRI} fragment off the \textit{hprt} minigene. (3) A 335 bp \textit{XhoI-HindIII} random primed fragment of the \textit{hprt} minigene.

Figure II.2b  \textit{hprt} minigene probes for Southern analysis from pBluescript KS + phagemid.

(4) A 335 bp \textit{XhoI-HindIII} fragment of the \textit{hprt} minigene ligated into Bluescript phagemid, digested with \textit{HindIII} and then specifically primed off the T3 promoter. (5) Same as (4) but digested with \textit{XhoI} after specific priming off the T3 promoter. (6) Single primed probe off the 3' end within the 335 bp \textit{XhoI-HindIII} fragment of the \textit{hprt} minigene giving a 330 bp probe.
μg of the total digest was electrophoresed on a 0.8% agarose gel to ensure that digestion was complete. The remaining 10 μg was size-fractionated by electrophoresis on an 0.8% agarose gel at 22V for 12 h. The DNA was degraded with 0.25 M HCl with rocking for 30 minutes and then placed into distilled water. The 16 h capillary blot transfer to a Hybond-N' membrane (Amersham Life Sciences, Oakville, ON) was accomplished with 0.4 M NaOH alkali solution. Membranes were prehybridized for up to 4 h in 50 μg ml⁻¹ denatured salmon DNA, 6 x SSC, 5 x Denhardt's solution, 0.5% (w/v) SDS and 50 mM sodium phosphate. Hybridization was performed by adding 50 μl (50 μCi) of ³²P-α-deoxycytidine triphosphate (dCTP) (Amersham Multiprime DNA labelling kit, Amersham Life Sciences, Oakville, ON) labelled and denatured probe to the prehybridization solution. The labeled probe was allowed to anneal for 12 to 16 h at 65°C. For each experiment, incorporation of radiolabelled nucleotide was 50-70%.

Hybridized membranes were washed twice with two 15 min changes of 2 x SSC + 0.1% SDS at 65°C followed by two 30 min washes with 0.16 x SSC + 0.1% SDS at 65°C.

Autoradiography was performed by exposing a storage phosphor intensifying screen to the membrane for 24 hours and scanning the image on phosphoimager SI scanner, version 3.51 (Molecular Dynamics, Sunnyvale, CA, USA).

2.5.7 Slot-Blot Analysis

Procedures employed in this protocol are as described by Bennett et al. (1994). Samples of RNA (100 μl), extracted according to the RNA extraction protocol (section 2.5.4), were mixed with 10 μl of 37% formaldehyde and then heated for 30 min at 65°C.
Just prior to transfer to the slot-blot apparatus, 100 \( \mu l \) of 5 M KCl at 55°C, was added to each sample. Samples were loaded onto the slot-blot apparatus with 2.5 M KCl hydrated Hybond-N membrane and transfer was completed over a period of 30 min in the presence of a flow of warm air. The wells were washed with 200 \( \mu l \) of 10 x SSC and the membrane was removed and air dried, heated to 80°C for 20 min, and irradiated with UV light for 3 min. Prehybridization and hybridization procedures are described by Chen and Birnboim (1993). For detection of neo expressing clones, a random primer labelling kit (Pharmacia, Piscataway, NJ) was used to incorporate \([^{32}P]dCTP\) into the 2.4 kb \(Bam\) HI / \(Hind\) III fragment of the pSV2 plasmid containing the neomycin resistance gene (Southern and Berg, 1982). Hybridized membranes were air dried and then exposed to preflashed X-ray film (Kodak) for 93 or 47 h at -100°C.

2.5.8 Polymerase Chain Reaction (PCR) and Reverse Transcription PCR (RT-PCR)

PCR amplification of DNA sequences was conducted for 35 cycles, using a programable thermal controller (M. J. Research Inc., Watertown, MA). All PCR reactions were carried out in 50\( \mu l \) and were overlaid with 30\( \mu l \) of silicon oil. The PCR mixtures contained DNA template (at concentrations specified in Figure legends), 20 pM of each primer, 200 \( \mu M \) dNTP’s, 2 mM MgCl\(_2\) and PCR buffer (20 mM Tris-HCl, 50 mM LCl). DNA Polymerase (3 U \(Taq\), DNA Polymerase, Native, Gibco BRL) was added after the initial denaturation of the genomic DNA at 95°C for 5 min. Each PCR cycle consisted of denaturation at 95°C for 30 s, annealing at 55°C for 45 s, and
extension at 72°C for 90 s. After the last cycle, the samples were incubated for 5 min at 72°C, than kept at 4°C until transfer to -20°C storage. A portion of the PCR product was subjected to electrophoresis on 0.8% agarose gel, stained with ethidium bromide and visualized under UV light. Molecular weight markers were specified in each experiment.

RT-PCR was performed on RNA samples extracted as described in section 2.5.4. To 1 μl of RNA (500 ng μl⁻¹) was added 11 μl of sterile H₂O and 10 pM of reverse primer (1 μl) and then samples were incubated at 70°C for 5 min. Samples were chilled on ice for 5 min, and then 5x RT buffer (4 μl, Gibco, BRL, Life Sciences), 2.5 mM each dNTP (4 μl), and 100 mM DTT (2 μl) were added. After equilibration of the samples at 37°C, 1μl (5 units) of Superscript reverse transcriptase (Gibco, BRL, Life Sciences) was added, the mixtures incubated at 42°C for 1 h, followed by a 30 min incubation at 50°C. The RT product (5 μl) was amplified by 30 cycles of PCR reaction, as described above, and then a 3% Metaphor agarose gel was used to separate the resulting products using a 100 bp ladder as a marker.

2.5.9 DNA Sequencing

DNA double-strand sequencing of the hprt minigene XhoI/HindIII fragment (see Figures II.1 and II.2b) ligated within the pBluescript K/S + phagemid, was performed manually by the dideoxy chain termination method using the T7 Polymerase Kit (Pharmacia Inc., Piscataway, MD, USA).
2.6 CYTOGENETIC ANALYSIS

2.6.1 Metaphase Spreads

To logarithmically growing TK6 cells, colcemid (Gibco BRL) was added to a final concentration of 50 ng ml\(^{-1}\) and then cultures were harvested by centrifugation 2 h later. 1 x 10\(^6\) cells were gently pelleted (300 x g) and resuspended in 8 ml of 75 mM hypotonic KCl for 12 to 15 min 37°C. Cold fixative (2 ml, methanol/acetic acid 3:1 (v/v)) was added to the suspensions and the cells were again collected by centrifugation (8 min, 200 x g). Cells were resuspended 3 times in fresh fixative, allowing at least 30 min between fixations. To prepare chromosome spreads, the fixed cells were dropped onto clean microscope slides and allowed to spread under 70% humidity. Slides were stained in 4% Giemsa (Gibco BRL) in PBS for 5-7 min or stored at -20°C in a N\(_2\) atmosphere for use in fluorescence in situ hybridization (FISH) experiments.

Mouse fibrosarcoma cell lines were cultured and fixed similarly to allow harvesting of metaphase cells; however, the slides were prepared and analysed by Dr. J. Breneman and Dr. J. Tucker at Lawrence Livermore National Laboratory, Livermore, California, U.S.A.

2.6.2 Fluorescent in Situ Hybridization (FISH)

Many variations of the basic protocol described by Blakey et al. (1993 and 1995) were attempted to localize the transfected hprt minigene. The basic protocol used a number of different hprt biotinylated DNA probes, both nick translated or randomly primed, to hybridize to metaphase chromosomes from a few TK6 hprt transfectants.
Metaphase spreads were treated with RNase (100 μg ml⁻¹ in 2 x SSC) for 90 min at room temperature and pepsin (0.005% in 10 mm HCl) for 10 min at 37°C. The slides were washed in 2 x SSC and fixed for 10 min at room temperature in PBS containing 2% formalin and 50 mM MgCl₂. The slides were placed in 70% formamide for 2 min at 72°C to denature the DNA, followed by dehydration in ethanol and pre-hybridization at 45°C in a mixture containing 50% formamide, 10% dextran sulfate, 0.56 ng carrier DNA, and 1 x Denhart's solution. The hybridization mixture was the same as for the pre-hybridization plus 0.1 to 100 ng of denatured biotinylated probe was added and the hybridization reaction was allowed to take place at 37°C for 16 to 18 h. After hybridization, the slides were washed three times in 50% formamide for 2 min, twice in 2 x SSC for 2 min, and twice in NP-40 buffer (0.1 M sodium phosphate, 0.05% Nonidet P-40, pH 8) for 2 min. Slides were stained for 20 min by 5 μl of fluorescein-avidin (25 ng μl⁻¹; Vector Labs, Burlingame, CA). Prior to a 20 min amplification staining with biotinylated anti-avidin (5 ng μl⁻¹, Vector Labs, Burlingame, CA) slides were washed twice in NP-40 buffer for 2 min, followed by another 20 min staining step with fluorescein-avidin. Chromosomes were counterstained for 20 min with DAPI (0.67 μg ml⁻¹ 4′,6′-diamidino-2-phenylindole, Boerhringer Mannaheim, Montreal, PQ) and mounted in Antifade (Vectashield, Vector Labs, Burlingame, CA). Images of FITC (green) and DAPI (blue) staining chromosomes were taken separately by using a Zeiss Axioskop 20 microscope fitted with CytoVision Probe system (Applied Imaging, Pittsburgh, PA, USA) in Dr. D. Blakey’s laboratory as described in (Blakey et al., 1995).
CHAPTER 3

IN VITRO AND IN VIVO GENOMIC INSTABILITY OF ENDOGENOUS hprt GENE IN MOUSE FIBROSARCOMA CELL LINES

3.1 INTRODUCTION

The objective was to develop a mouse transplantable (in vivo / in vitro) tumour cell line highly sensitive to mutational events at a heterozygous hprt marker gene. Our laboratory was interested in using this model to identify tumour-associated mutagenic factors. The model was designed to induce tumours within a syngeneic animal and to monitor damage acquired by the tumour cell in vivo at a genetic marker capable of detecting large and small scale mutational events. The development of this model would provide a means of identifying those factors within the tumour environment that could contribute to genomic instability and to the eventual progression of the tumour to a more malignant state.

The strategy was to develop a transplantable cell line with a heterozygous hprt gene marker that would have increased sensitivity in detecting damage from clastogenic agents such as radiation, a known inducer of DNA double strand breaks and large scale mutational events, primarily deletions (Vrieling et al., 1985; Thacker, 1986; Fuscoe et al., 1986; Sankaranarayanan, 1991).
3.2 DERIVATION OF MC1A AND SUBLINES

3.2.1 BACKGROUND INFORMATION

The cell line selected for developing a mouse transplantable model, MC1A, was a mouse fibrosarcoma originally isolated from a male C57BL/6 mouse that had been subcutaneously inoculated with 500 µg 3-methylcholanthrene (Kadhim and Rees, 1984). MC1A-C1 is a variant capable of in vitro growth that arose spontaneously when MC1A cells were maintained in culture for several weeks. Eighteen 6-TG$^R$ (HPRT$^+$) mutants were eventually isolated from MC1A-C1 by a 1 h treatment with N-methyl-N-nitrosourea (MNU) (125 µM), followed by two months of incubation in the presence of 0.5 µM 6-TG. This observed difficulty in inducing 6-TG$^R$ mutants was unexpected since most published reports demonstrate a relatively easy induction of mutations at this normally hemizygous location. Derivation of MC1A-C1 6-TG$^R$ mutants was accomplished in Dr. Birnboim’s laboratory prior to initiation of this thesis. For complete summary and nomenclature of cells used in this chapter refer to Appendix 1.

MC1A-C1 fibrosarcoma is an anchorage-dependent cell line that grows as a monolayer with some focal piling. Mutant cells lacking HPRT (6-TG$^R$) can still be killed by a process known as ‘metabolic cooperation’, the passive acquisition of the toxic nucleotide 6-thioguanine monophosphate (6-TGMP) (Elion, 1989) through gap junctions of neighbouring cells that contain the active enzyme (Trosko and Chang, 1984). One solution to minimize the effects of metabolic cooperation is to grow cells at low density so that they do not make contact. To determine the maximum allowable cell density
(number of cells per plate), a reconstruction experiment was carried in which a known number of resistant cells was plated in the presence of increasing number of sensitive cells.

The tumourigenicity of 17 clones were also verified since the ultimate design of the experiment was dependent on the capacity of the selected clone to induce tumours in a syngeneic C57BL/6 mouse. The most tumorigenic clone was further enhanced for tumorigenicity by a second passage through the animal.

3.2.2 METHODOLOGY

3.2.2.1 Intercellular Communication. A reconstruction experiment for estimating survival of HPRT+ mutant in the presence of HPRT- wild type mouse fibrosarcoma cells was performed by plating 100 6-TG<sup>R</sup> (MC-TGR17) cells in 10 cm tissue culture dishes with increasing number of 6-TG sensitive (6-TG<sup>S</sup>) cells. Following 14 days of incubation in 6-TG, the resistant surviving colonies were scored as described in section 2.2.4.

3.2.2.2 Tumorigenicity Testing. Tumorigenicity of seventeen 6-TG<sup>R</sup> mutants was tested by monitoring the development of tumours (size) in syngeneic female mice following subcutaneous injection of 1 x 10<sup>6</sup> cells in 0.1ml PBS. For each clone, 3 injected animals were examined at regular intervals for up to 25 days. When tumours reached 1.5 cm in size, the animals were euthanised, and, in the case of MC-TGR17, cells were cultured from the excised tumour and re-injected into another 3 mice. These newly developed tumours were once again monitored for growth, excised at the size of 1 cm,
pooled and cultured for use in future experiments.

3.2.3 RESULTS

3.2.3.1 Intercellular Communication. Inconsistency in the number of HPRT\(^{-}\) viable colonies in an excess of HPRT\(^{+}\) non-viable cells was observed during selection in 6-TG. The possibility that metabolic cooperation (intertacellular communication) had an effect on the mutants being recovered was explored through a reconstruction experiment (Fig. III.1). The recovery of 6-TG\(^{R}\) colonies was unchanged in the presence of up to \(1 \times 10^{5}\) 6-TG\(^{S}\) (MC1A-C1) cells per 10 cm dish. Above this cell number, there was a clear decrease in recovery of 6-TG\(^{R}\) colonies such that, at \(1 \times 10^{6}\) cells, very few colonies were recovered.

3.2.3.2 Tumorigenicity Testing. Differences in the tumorigenicity of various MC-TG\(^{R}\) clones was observed in both the frequency of tumour induction and the rate of tumour growth. Table III.1 data presents the average tumour size per mouse for each different clone tested. One of the most tumorigenic clones (MC-TGR17) was recovered from a tumour and then re-injected into other mice in an attempt to further enhance tumourigenicity. Enhancement of tumorigenicity was detected after the second passage of clone MC-TGR17. Secondary tumours were excised, pooled and cultured for subsequent use (section 3.3).
Figure III.1  Effects of cell density upon metabolic cooperation.

100 6-TG<sup>R</sup> cells were added to varying concentrations of 6-TG<sup>S</sup> cells, and clonable cells were scored after a 14 to 21 day culture period. Each bar represents the mean of the number of 6-TG<sup>R</sup> surviving colonies from at least three different plates with the error bars representing the standard error of the mean. The first two bars represent the number of clonable 6-TG<sup>R</sup> cells from 100 cells seeded (± 6-TG). All other bars represent total clonable cells in the presence of 6-TG.
Table III. 1  Tumorigenicity of MC-TG<sup>8</sup> clones

<table>
<thead>
<tr>
<th>Clone</th>
<th>Day 8</th>
<th>Day 11</th>
<th>Day 14</th>
<th>Day 18</th>
<th>Day 21</th>
</tr>
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<tr>
<td>MC-TGR1</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<td>0.2</td>
</tr>
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<td>0.7</td>
<td>0.8</td>
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<tr>
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<td>0</td>
<td>0.1</td>
<td>0.5</td>
<td>0.7</td>
</tr>
<tr>
<td>MC-TGR11</td>
<td>0</td>
<td>0</td>
<td>0.2</td>
<td>0.3</td>
<td>0.8</td>
</tr>
<tr>
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<td>0.1</td>
<td>0.3</td>
<td>0.8</td>
<td>0.7</td>
<td>0.8</td>
</tr>
<tr>
<td>MC-TGR13</td>
<td>0.1</td>
<td>0</td>
<td>0.2</td>
<td>0.7</td>
<td>0.8</td>
</tr>
<tr>
<td>MC-TGR15</td>
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<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td>0.5</td>
</tr>
<tr>
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<td>1.3</td>
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</tr>
<tr>
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<td>0.6</td>
<td>1.3</td>
<td>1.5</td>
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</tr>
<tr>
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<td>0.7</td>
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<td>0</td>
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<td>0.7</td>
</tr>
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<td>0.5</td>
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</tr>
<tr>
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<tr>
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<td>0.5</td>
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<tr>
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<td>0.8</td>
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<tr>
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</tr>
<tr>
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<td>0</td>
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<td>1.0</td>
</tr>
<tr>
<td>MC1A-C1</td>
<td>0.13</td>
<td>0.6</td>
<td>1.0</td>
<td>1.5</td>
<td>+++</td>
</tr>
<tr>
<td>MC-TGR17</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+++</td>
</tr>
<tr>
<td>2&lt;sup&gt;nd&lt;/sup&gt; Passage</td>
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<td>1.0</td>
<td>1.3</td>
<td>+++</td>
<td>+++</td>
</tr>
</tbody>
</table>

Each clone was tested for tumorigenicity in three different mice. Data points represent an average tumour diameter (cm) / mouse. +++ The animals were sacrificed when tumour size exceeded a diameter of 1.0 cm.
3.2.4 SUMMARY

To successfully determine the contribution made by in vivo environmental factors to mutational events and tumour progression, MC1A-C1 cells had to be selected for growth under both in vitro and in vivo conditions, that is, it was necessary to select a transplantable and highly tumorigenic 6-TG\(^R\) mutant cell line. However, this proved very difficult despite treatment with a strong mutagen. The difficulty in recovering \(\text{hprt}\) mutants is now suspected to be due to the presence of multiple X-chromosomes and therefore a presence of multiple copies of the \(\text{hprt}\) gene (see chapter 3.4). This very low mutant recovery contrasted with previous publications (Evans et al., 1986a; 1986b), showing spontaneous mutations at the murine \(\text{hprt}\) locus at a frequency of 6 to 11 \(\times\) 10\(^{-6}\) mutants, and induced frequency of 15 \(\times\) 10\(^{-6}\) mutants per Gy after an acute exposure to \(\gamma\) radiation, or 320 \(\times\) 10\(^{-6}\) mutants at the \(D_{37}\) concentration of MNU.

Once some 6-TG\(^R\) mutants of MC1A-C1 were isolated, these could be used to test the hypothesis that metabolic cooperation between the mouse fibrosarcoma adherent cells was interfering in mutant recovery, and to establish conditions to minimize these effects. A reconstruction experiment examining the effects of increased cell density upon mutant cell viability was conducted. From the data of Figure III.1 it can be concluded that the toxic 6-TGMP nucleotide from HPRT\(^-\) cells could be detrimental to the viability of neighbouring HPRT\(^+\) cells. These findings established that mouse fibrosarcoma experiments had to be conducted at cell concentrations not exceeding 1 \(\times\) 10\(^5\) cells per 10 cm dish.

Having established appropriate culture conditions to minimize metabolic
cooperation, the next step in developing our model system was to recover a maximally tumorigenic 6-TG\textsuperscript{R} mutant clone. When the tumourigenicity of the 17 clones were compared (Table III.1), differences were found both in lag times and sizes of the tumours. One of the most tumorigenic clones MC-TGR17 was selected for future experiments. This clone was passaged through a mouse for a second time to ensure, and potentially enhance, tumorigenicity by \textit{in vivo} clonal selection.
3.3 SELECTION OF HAT$^R$ CLONES

3.3.1 OBJECTIVES

The experimental strategy was to integrate a transgene into a 6-TG$^R$ mutant cell (MC-TGR17), at different chromosomal locations, to be used as a marker of genomic instability. A hprt minigene was excised from the pHPT37-D2 plasmid. To confirm that this human minigene was functional upon transfection into mouse cells, the SP2/0 mouse myeloma cell line (ATCC), with known deficiency at the endogenous hprt gene locus, was electroporated with the excised minigene and a number of phenotypically reverted cells were recovered. These HAT$^R$ transfectants were isolated at the beginning of this project in Dr. Birnboim’s laboratory, suggesting that the human hprt minigene was capable of proper expression upon transfection into mouse cells. One clone was examined by Southern hybridization analysis for the presence of the minigene and a hybridization product of appropriate size was found suggesting that the transfection was successful. This same clone was further examined for the spontaneous and induced stability of transgene expression.

Section 3.2 describes the derivation of an in vivo / in vitro mouse tumorigenic 6-TG$^R$ cell line. Since the objective of this thesis was to create a transplantable cell line with a sensitive marker of mutational events, the next step was to transfect this 6-TG$^R$ mutant with a hprt minigene and then to select a number of clones with the desired HAT$^R$ phenotype.

Since the final objective of this project was to develop an in vivo / in vitro model
to detect mutational events, it was decided to explore the possibility of establishing an alternative tumour environment for the selected cell line. One clone (MC-TGS17-51), was serially passaged through the mouse peritoneal cavity, with the objective of developing an ascites tumour that could be used as a new model system for detecting mutational events within a tumour environment. One potential problem that presented itself early in the passaging of the selected cell line through the mouse peritoneal cavity was the possibility that some of the recovered cells could be contaminating host cells. To ensure that the number of mutants recovered from the excised mouse tumours would reflect the mutation frequency from the tumour fibrosarcoma cell population only, and not be diluted by the presence of infiltrating mouse host cells, a *neo* construct was introduced into the MC-TGS17-51 cell line to provide a means of selectively recovering the desired population of tumour cells.

3.3.2 METHODOLOGY

3.3.2.1 Stability of *hprt* Transgene expression in the Mouse Myeloma Cell

*Line*. A reconstruction experiment for mouse myeloma cells was performed by plating a total of 30 6-TG<sup>R</sup> SP2/0 cells per 96 multiwell tissue culture plate with 1 x 10<sup>5</sup> SPTG<sup>S</sup> cells in the presence of 6-TG. As described in section 2.2.2, plates were incubated and wells with viable cells were scored after an appropriate culture period. Stability of the HAT<sup>R</sup> phenotype in the SP2/0 transfectants was monitored for spontaneous and Co<sup>60</sup> γ-ray induced reversion to 6-TG<sup>R</sup> phenotype. Cells cultured for 4 weeks in non-selective media or cells cultured for 1 week after irradiation in non-selective media were 6-TG
challenged in 96 well plates. As before, the wells were scored for growth after appropriate culture time.

3.3.2.2 Attempted Transfection of 6-TG\textsuperscript{R} Mouse Fibrosarcoma and Characterization of Selected Clones. Knowing that the human \textit{hprt} minigene construct is functional in the mouse myeloma cells (work done prior to this thesis), it was our intent to transfect the HPRT\textsuperscript{-} mouse fibrosarcoma cell line with this same construct. Following a second animal passage of the most tumorigenic cell line (MCTGR-17) derived in section 3.2, the pooled sample was subjected to electroporation with human \textit{hprt} minigene as described in section 2.3.2. HAT\textsuperscript{R} clones were selected and characterized for their stability of \textit{hprt} gene expression. Preliminary screening for \textit{hprt} gene expression (single time point) of 50 out of the 54 HAT\textsuperscript{R} clones maintained in non-selective medium for 26 to 35 days was conducted by challenging the clones to grow in 6-TG. A more detailed estimate of genomic instability at the \textit{hprt} gene locus was performed by examining the spontaneous mutation rates of 14 clones. Five of these 14 clones were also irradiated with Co\textsuperscript{60} \textgreek{y}-rays, to determine the ability of ionizing radiation to induce mutation at the marker gene. These same 5 clones were also tested for differences in their doubling times. The above experiments were not corrected for plating efficiencies. Mutant selection and irradiation protocols are presented in sections 2.2.2 and 2.4.2 respectively.

3.3.2.3 Relative Growth Rates of Mutants and Parental Cells. In calculating mutation frequencies, it was assumed the growth rates, and thus the doubling times, of the 6-TG\textsuperscript{R} mutants and the parental HAT\textsuperscript{R} clone would be the same. This assumption allowed
estimation of ratios of mutant cells to wild type cells in cultures maintained over a period of time. To test this assumption, and to ensure that selection pressure was not biasing growth of mutants, a reconstruction experiment was conducted to establish mutant survival capabilities of a pool of 6 independent, spontaneously arising 6-TG\textsuperscript{R} mutants added to a population of HAT\textsuperscript{R} cells. Five separate tissue culture plates were seeded with $3 \times 10^3$ (6-TG\textsuperscript{R}) mutants and $1 \times 10^6$ HAT\textsuperscript{R} (6-TG\textsuperscript{S}) MC-TGS17-51 cells. Immediately after seeding, a sample of $1 \times 10^5$ cells, from each mixed population, was removed and challenged for 6-TG resistance. The remaining mixed population was maintained under non-selective conditions for 25 days, with subculturing as required. At the end of the 25 days, another 5 plates (one from each of the five mixed cultures) were set up in an identical fashion to select for 6-TG\textsuperscript{R} mutants. At each point of challenge, assays for plating efficiency were used to correct for the number of viable mutant colonies recovered following incubation.

3.3.2.4 *Attempt to Establish Ascites Tumour.* Attempts were made to develop an ascites tumour from MC-TGS17-51, a clone derived from MC1A-C1, a mouse fibrosarcoma cell line. Mice were inoculated at two different sites in the peritoneal cavity with a total of 1 ml of $1 \times 10^6$ HAT\textsuperscript{R} cells in PBS. After 21 days, mice were euthanised and their peritoneal cavities flushed with 3 ml of PBS and the recovered cells (pooled samples from all animals) were plated onto either tissue culture or bacteriological 10 cm plates in the presence of non-selective DMEM. The plates were cultured, and recovered cells were reinjected into mouse peritoneal cavity. This cycle of injection and recovery was repeated 6 times, with at least three animals each time.
3.3.2.5 Introduction of neo as a Selectable Marker. In response to the concern that contaminating mouse host cells (mutant or wild type), might interfere with the calculation of the mutation frequency, clone MC-TGS17-51 was infected with a neo gene construct as a selectable marker (section 2.3.1). Slot blot analysis was performed on twelve different clones cultured in G-418. Experimental protocols were previously described in sections 2.3.1 and 2.5.7. A pool of G418 resistant cells was sub-cloned by J.K.S. Sandhu to isolate MN cell lines which were used in her thesis project to examine the effects of nitric oxide on mutation induction under in vivo and in vitro conditions.

3.3.3 RESULTS

3.3.3.1 Stability of hprt Transgene Expression in the Mouse Myeloma Cell Line. In a reconstruction experiment testing survival of 30 6-TG<sup>R</sup> cells in the presence of 1 x 10<sup>5</sup> HAT<sup>R</sup> mouse myeloma cells, 2 different experiments demonstrated a plating efficiency of 50 ±15% and 60 ±10% respectively. All screening of mouse myeloma mixed populations for spontaneous and induced mutant recovery was performed at cell densities of 1 x 10<sup>5</sup> cells/ 96 well plate. Examination of spontaneous mutation frequencies of the HAT resistant SP2/0 (SPTG<sup>5</sup>) clones to 6-TG resistance yielded an unexpectedly high frequency of gene loss. After a 4 week culture period in non-selective medium, 6 SPTG<sup>5</sup> clones accumulated SPTG<sup>R</sup> revertants at ≥1 x 10<sup>-3</sup> per 10<sup>5</sup> cells plated. An experiment to assess the induction of mutants by Co<sup>60</sup> γ-rays was hampered by this high background. When cultures were tested 7 days after irradiation, total (induced plus spontaneous) mutation frequencies ranged from 1 x 10<sup>-4</sup> to 1.4 x 10<sup>-3</sup> per Gy. However,
because of the high background, it was difficult to determine the significance of these values.

3.3.3.2 Characterization of HAT\textsuperscript{R} Mouse Fibrosarcoma Clones - Spontaneous

Mutations. Following electroporation of 8 \times 10\textsuperscript{6} MC-TGR17 cells in the presence of the hprt minigene, 54 HAT resistant mutants were recovered. Based on the studies using the SP2/O cells, these mutants were postulated to be stable transfectants of the human hprt minigene, although the number of transfectants was low. These “transfectants” were further characterized for the presence of the transgene product by RT-PCR as discussed in section 3.4. Preliminary analysis of 50 out of 54 clones selected in HAT demonstrated considerable variation in the apparent stability of expression of the hprt gene (Fig. III.2). Thirty percent of the clones demonstrated a highly unstable phenotype where >100 mutants per 1 \times 10\textsuperscript{5} viable cells were detected following a 26 to 35 day incubation under non-selective conditions. The remaining 70% of the clones also had a high spontaneous mutation frequency, with the most stable of these demonstrating more than 5 mutants per 10\textsuperscript{5} cells during the same culture period. A more detailed look at 14 spontaneous 6-TG\textsuperscript{R} mutants arising over a period of 4 weeks, allowed an estimate of mutation rates; they demonstrated considerable variability in hprt stability (Figures III.3 and III.4). Twelve of 14 clones demonstrated an increase in the number of 6-TG\textsuperscript{R} colonies as a function of time in non-selective medium (Table III.2 and Table III.3). Clones MC-TGS17-20 and MC-TGS17-50 demonstrated a slight negative slope suggesting a decline in the number of 6-TG\textsuperscript{R} colonies recovered with time. The remaining 12 clones varied in their hprt mutation rates from 0.3 \times 10\textsuperscript{-5} per day for the
Figure III.2  Spectrum of *hprt* spontaneous reversion frequencies in 50 clones selected with HAT resistant phenotype.

Each bar represents the number of 6-TG<sup>+</sup> cells per $10^5$ cells tested. A single data point is represented in each bar. The test population was cultured for 26 to 35 days in non-selective medium prior to challenge. * Colonies selected for more detailed studies.
AFTER 26 TO 35 DAYS IN NON-SELECTIVE MEDIUM
Figure III.3  Spontaneous generation of 6-TG\textsuperscript{R} mutants

Ten different MC-TG\textsuperscript{S} clones were grown in non-selective medium until, at three different time points, a fraction was sampled for challenge with 6-TG. Each clone was independently tested for generation of 6-TG\textsuperscript{R} mutants on day 0, 7 and 27, and the results were plotted as the mean values of at least three test plates ± standard error of the mean.

Figure III.4  Spontaneous generation of 6-TG\textsuperscript{R} mutants in five HAT\textsuperscript{R} clones as a function of time.

Five different MC-TG\textsuperscript{S} clones were grown in non-selective medium for up to 35 days. At regular 7 day intervals, a fraction of cell population was tested for generation of 6-TG\textsuperscript{R} mutants. Each bar represents the mean value of 3-9 test plates ± standard error of the mean.
Table III.2  Spontaneous mutation rates for 10 MC-Tg<sup>5</sup> clones

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<tr>
<td>mutants / 10&lt;sup&gt;5&lt;/sup&gt; cells / day</td>
<td>18.7</td>
<td>10.6</td>
<td>6.6</td>
<td>3.5</td>
<td>1.4</td>
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<tr>
<td>Mutants / 10&lt;sup&gt;5&lt;/sup&gt; cells / day</td>
<td>1.3</td>
<td>0.9</td>
<td>0.3</td>
<td>-0.1</td>
<td>-0.6</td>
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Mutation rates were calculated from linear regression analysis of the spontaneous mutation frequency curves presented in Figure III.3. In all cases, except for clones MC-TGS17-20 and -50, the r values exceeded 0.97.

Table III.3  Spontaneous mutation rates for 5 MC-Tg<sup>5</sup> clones

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<tr>
<td>Mutants / 10&lt;sup&gt;5&lt;/sup&gt; cells / day</td>
<td>10.3</td>
<td>1.6</td>
<td>1.1</td>
<td>1.0</td>
<td>0.3</td>
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Mutation rates were calculated from linear regression analysis of the spontaneous mutation frequency curves presented in Figure III.4. In all cases, the r values exceeded 0.90.

most stable clone MC-TGS17-51, to 19 x 10<sup>5</sup> per day for clone MC-TGS17-17, which had the least stable phenotype.

3.3.3.3 Characterization of HAT<sup>+</sup> MouseFibrosarcoma Clones - Induced

Mutations. Five clones were irradiated to determine their sensitivity to mutation induction. They demonstrated considerable difference in the number of recovered mutants (Fig. III.5). In each case, the induced number of mutants was calculated as the
Figure III.5  Induction of 6-TG$^R$ mutants by ionizing radiation

The number of 6-TG$^R$ radiation induced mutants was determined following exposure of five MC-TG$^S$ clones to 200, 400 and 800 rads. Each bar represents the difference of the means, between the total number of mutants after irradiation and the spontaneous number of mutants arising in unexposed controls (0 rads), over the same time interval. Each mean is representative of at least three data points.
difference between the total number of mutants after irradiation and the spontaneous number of mutants arising in unexposed controls over the same time interval. Linear regression analysis of the dose response curve for the five mutants, gave correlation coefficients (r values) between 0.909 and 0.995, and demonstrated a range in mutation rates of $8.6 \times 10^{-5}$ cells per Gy to $59.4 \times 10^{-5}$ cells per Gy, (Table III.4). Doubling times were presented in the same Table, demonstrating no detectable differences between the clones.

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<tr>
<td>Mutants / $10^5$ cells / Gy</td>
<td>8.6</td>
<td>20.7</td>
<td>22.4</td>
<td>27.6</td>
<td>59.4</td>
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<tr>
<td>Doubling Times (h)</td>
<td>18.3</td>
<td>19.5</td>
<td>17.9</td>
<td>18.9</td>
<td>19.8</td>
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Mutation rates were calculated from linear regression analysis of the mutation frequency curves presented in Figure III.5.

3.3.3.4 Relative growth rates of mutants and parental cells. A reconstruction experiment examining the proliferation rate of 6 different $6\mathrm{-TG}^R$ mutants supported the assumption that at least under in vitro conditions no selective advantage or disadvantage was biasing the recovery of spontaneously arising mutants. The mean number of surviving $6\mathrm{-TG}^R$ colonies (corrected for plating efficiency) was $100 \pm 9$ immediately following mixing and $121 \pm 15$, 25 days later, a non-significant difference (these numbers are adjusted for the newly arising $6\mathrm{-TG}^R$ mutants in the population of $1 \times 10^5$ cells over a period of 25 days).
3.3.3.5 Attempts to Establish Ascites Tumour From MC-TGS17-51

**Fibrosarcoma Cells.** Establishment of an ascites tumour from a mouse fibrosarcoma clone (MC-TGS17-51) was pursued over 6 cycles of injection and recovery in hopes of inducing an anchorage-independent variant. Recovered intraperitoneal flush fluid which was incubated *in vitro* for 3 to 5 days in either bacterial or tissue culture plates, yielded viable cells from the latter cultures only. Subsequent culturing of the supernatant from the tissue culture plates resulted in a sequential decrease in the growth of adherent cells with no observed proliferation of anchorage independent cells. This approach was therefore abandoned.

3.3.3.6 Introduction of neo Selectable Marker. Successful infection of MC-TGS17-51 cells with the *neo* marker gene was confirmed by slot-blot analysis (Fig. III.6). RNA from 11 clones selected in G418 hybridized to the *neo* probe and gave a strong signal while RNA isolated from parallel G418 sensitive clones did not.

3.3.4 SUMMARY

SP2/0 mouse myeloma cell line, known to be HPRT deficient, was used to verify that pHPT37-D2 construct carried a functional *hprt* minigene. Electroporation of SP2/0 mouse myeloma cells with this construct produced HAT \(^R\) clones, demonstrating that a human minigene was functional in a mouse cell line.

Electroporation of \(8 \times 10^6\) MC1A-C1 mouse fibrosarcoma 6-TG resistant cells resulted in the isolation of 54 HAT \(^R\) clones (calculated efficiency of \(1/6 \times 10^4\)). These HAT resistant clones were assumed to be transfectants although subsequent experiments
Figure III.6  Slot-blot analysis of MC-TGS17-51 sub-clones showing expression of the neo gene marker.

RNA, isolated from 12 control sub-clones and 12 G418 resistant sub-clones, was probed with neo in two parallel experiments. In each experiment, the first 12 slots were loaded with RNA from control cells, and into the remaining 12 slots the RNA from G418 resistant cells was applied. 11 of the G418 resistant sub-clones demonstrated the presence of a functional neo gene.
(Chapter 3.4) suggested that most, if not all of them were phenotypic revertants.

A wide difference (at least 60 fold) in the spontaneous mutation rate was observed in the original 54 “transfectant” clones derived from the MC-TGR17 clone. However, it was expected that at least some of the clones would display a mutation frequency at the locus of the “transfected” hprt minigene in the order of $6 \times 10^6$ mutants per viable cell, similar to that observed at the endogenous mouse gene locus (Evans et al., 1986b). Linear regression analysis of spontaneous mutation data at day 7 (Figures III.3 and III.4), uncorrected for plating efficiency, estimated the most stable clone (MC-TGS17-51) to have a frequency of approximately $22 \times 10^6$ mutants per cell. Correction of this data by the average observed plating efficiency of 50% gave a spontaneous mutation frequency of about $44 \times 10^6$, at least four fold higher than that previously reported at the mouse endogenous hprt hemizygous locus but within the range observed at the endogenous heterozygous tk gene locus (Evans et al., 1986b). This clone was selected for further study since it demonstrated the most stable hprt gene expression under normal culture conditions.

Characterization of five selected clones, including MC-TGS17-51, was conducted by examining the mutation frequency induced by exposure to the clastogenic Co$^{60}$ γ-rays. All five clones tested demonstrated a dose-dependent rate of mutation induction (Fig. III.5) ranging between 86 mutants per $10^6$ cells per Gy to 594 mutants per $10^6$ cells per Gy, uncorrected for plating efficiency. The differences in mutant accumulation between clones could not be explained on the basis of growth rate differences. The average radiation induced mutation frequencies of the 3 subclones were similar to those described.
for the heterozygous tk gene (Evans et al., 1986b). Considering the signal to noise ratio
(induced to spontaneous mutations) and the observation that the spontaneous mutation
frequency was similar to that observed by Evans et al. (1986b) at the endogenous tk gene,
clone MC-TGS17-51 was selected as the clone most likely to be a sensitive detector of
induced mutational events.

Repeated attempts to establish an anchorage-independent ascites tumour cell from
the mouse fibrosarcoma cell line, MC-TGS17-51, were unsuccessful. One possible
alternate route in recovering an anchorage-independent variant might be to produce a cell
hybrid between an anchorage dependent and an anchorage independent cell and select for
those expressing an appropriate phenotype (Gourdeau and Walker, 1994).

Transfection of the neo gene into the MC-TGS17-51 clone allowed the injected
tumour cells to be differentiated from the host cells following the excision of the
developed tumour. The ability to differentiate the host cells from the injected test cells
ensured the in vivo mutation frequency could be corrected to specifically reflect the
damage incurred by the test cells.

Clone MC-TGS17-51 demonstrated spontaneous and induced mutation
frequencies similar to those observed in the endogenous heterozygous tk marker gene
(Evans et al., 1986b). As a consequence, these cells can be used to both induce tumours
and to monitor mutational events taking place within the in vivo environment with the
same power of detection as an endogenous heterozygous gene marker. The work
described in the next two sections further characterizes the selected clone, MC-TGS17-
51, and firmly demonstrates its capacity as a vehicle for the recovery of in vivo mutations.
3.4 CHARACTERIZATION OF MC-TGS17-51 (HAT\textsuperscript{R}) CLONE

3.4.1 OBJECTIVES

Despite the identification of a cell line with the required sensitivity for detecting both spontaneous and induced mutational events, further characterization of the cells was necessary to better understand the process of mutant induction. As described in section 3.2, the difficulty in initially inducing mouse fibrosarcoma 6-TG\textsuperscript{R} mutants was examined further by comparing the mutant recovery after irradiation for both the parental MC1A-C1 and the derived MC-TGS17-51 clone. To ensure that the lack of recovery of MC1A-C1 mutants was not a consequence of radiation killing, the two cell lines were compared for viability after irradiation, and survival-dose response curves were established and compared.

The observed difficulty in recovering MC1A-C1 6-TG\textsuperscript{R} mutants could possibly be explained by the presence of multiple X-chromosomes and consequently of more than one functional hprt gene. To test for this, the karyotype of the mouse cell lines were studied as soon as appropriate mouse probes were available. Collaborative efforts with Dr. J. Tucker's laboratory, Lawrence Livermore National Laboratories, and the use of their newly developed mouse X-specific probes revealed a possible explanation for the difficulty of 6-TG\textsuperscript{R} mutation induction.

Due to very low initial recovery of 6-TG\textsuperscript{R} MC1A-C1 mutants (section 3.2), the
karyotypic analysis from Dr. J. Tucker's laboratory, and the unexpectedly low yield of recovered transfectants after electroporation (section 3.3), it was necessary to further characterize those clones recovered in HAT that expressed the expected phenotype. These HAT\textsuperscript{R} clones could be explained by either the successful transfection of a mutant with the \textit{hprt} minigene, or alternatively, by a process that allowed an inactive endogenous \textit{hprt} gene(s) to be reactivated. To explore the latter possibility, the 6-TG\textsuperscript{R} clone recovered from MC1A-C1 was monitored for reversion. Counts and Goodman (1995) suggested that mouse cells isolated from C57BL/6 mice are capable of maintaining normal levels of methylation, similar to that observed in human cells. Thus, a MNU / 6-TG induced mutation at the \textit{hprt} locus could be reactivated by hypomethylation under \textit{in vivo} conditions to express the endogenous gene. It is relevant to note that MNU is a methylating agent known to induce mutations that can be repaired (Akagi et al., 1993; Wurdeman et al., 1993; Shibata et al., 1994). Southern analysis, PCR and RT-PCR analysis were performed on some of the HAT\textsuperscript{R} clones to determine whether the functional \textit{hprt} gene was of murine or human transgene origin. Also, the possibility was examined that some of the highly mutable HAT\textsuperscript{R} clones (MC-TGS17-17 and -39) could be unstable transfectants. These studies led to a better understanding of the genotype of the selected MC-TGS17-51 clone.

3.4.2 METHODOLOGY

3.4.2.1 \textit{Mutation Induction}. The number of recovered mutants, after various doses of Co\textsuperscript{60} \textit{γ} irradiation, was determined for MC1A-C1 and MC-TGS17-51 clones
following a one week period of expression in non-selective medium prior to a 6-TG selective challenge, as described in sections 2.2.4 and 2.4.2. Plating efficiencies were estimated at the same time. Survival curves for the two mouse fibrosarcoma cell lines were established by seeding a known number of cells per 10 cm dish immediately following irradiation and allowing sufficient culture time for the viable cells to develop into scorable colonies (up to 28 days).

3.4.2.2 Karyotyping. Karyotype analysis of the three generations of the mouse fibrosarcoma cell lines (MC1A-C1, MC-TGR17, and MC-TGS17-51) was performed in Dr. J. Tucker's laboratory, Lawrence Livermore National Laboratory. Cultured cells arrested at metaphase by colcemid were exposed to hypotonic conditions (0.075 M KCl) and fixed (methanol:glacial acetic acid 3:1) for the preparation of metaphase spreads, then sent at room temperature to Drs. J. Breneman and J. Tucker at Lawrence Livermore National Laboratory. Identification of X-chromosomal material in these mouse cells was accomplished by hybridization with a biotinylated X chromosome-specific composite DNA probe (Breneman et al., 1993). Hybridization and detection of bound probe was accomplished with two layers of avidin-FITC as described (Breneman et al., 1995). Representative cells were photographed with Kodak Ektachrome 400 film on a Zeiss Axiophot photo microscope (Fig. III.7).

3.4.2.3 Stability of a 6-TG<sup>R</sup> Mutant. Stability of 6-TG<sup>R</sup> phenotype expression was monitored in the MNU / 6-TG derived HPRT<sup>-</sup> (MC-TGR17) mutant. This clone was challenged to grow in HAT selective medium, thus establishing the spontaneous reversion frequency to HAT<sup>R</sup> phenotype from the 6-TG<sup>R</sup> phenotype. Cells isolated both
Figure III.7  Detection of X chromosome-specific sequences in MC1A-C1 and MC-TGS17-51 cells by fluorescence *in situ* hybridization.

Metaphase spreads of MC1A-C1 (top) and MC-TGS17-51 (bottom). All three cell lines tested (including MC-TGR17 not represented in this figure) demonstrated a similar fluorescence banding pattern to X chromosome-specific sequences. The large arrows locate multiple X-chromosomes. Small arrows point to examples of interstitial and centromeric X-specific sequences (Wilkinson et al., 1995).
pre- and post-passage through the mouse host were examined for spontaneous reversion
to HAT resistance. MC-TGR17 cells that had not been passaged through the mouse were
cultured for four weeks in non-selective medium, sub-cultured to 10 plates of $10^4$ cells
per plate in HAT medium, and then monitored for resistant colonies, as described in
section 2.2.4. A parallel experiment was conducted using MC-TGR17 cells which were
recovered after two passages through the mouse, with the total culture time (in vitro plus
in vivo), without selection pressure, of seven weeks. This recovered cell population was
also tested for spontaneously arising HAT$^+$ colonies following one additional week of
culture in non-selective medium.

3.4.2.4 Origin of the Functional hprt Gene. MC1A-C1, MC-TGR17, and MC-
TGS17-51 mouse fibrosarcoma clones were analysed by Southern analysis. PCR
experiments, to detect the presence of the human hprt transgene, were carried out on the
DNA isolated from different generations of the mouse fibrosarcoma cells MC1A-C1,
MC-TGR17, MC-TGS17-51, other MC-TGS clones, and MN11, the clone selected after
neo infection of MC-TGS17-51. In these experiments pHPT37-D2 plasmid containing
the human hprt minigene was used as a positive control for PCR amplification. RNA
extracted from MC1A-C1, MC-TGR17, and MN-11, were subjected to RT-PCR to
further examine the origin of the functional hprt in the different clones. Human fibroblast
RNA, kindly provided by Dr. S. Bennett, was used as a control for the reaction.

3.4.3 RESULTS

3.4.3.1 Mutation Induction by Ionizing Radiation. MC-TGS17-51 clone was
compared to MC1A-C1 parental cell line in terms of sensitivity to induction of 6-TG\textsuperscript{R} mutants by Co\textsuperscript{60} γ-rays (Fig. III.8). A dose dependent increase in 6-TG\textsuperscript{R} colonies was observed for MC-TGS17-51 while no 6-TG\textsuperscript{R} colonies were detected in three independent experiments involving a total of 3.4 x 10\textsuperscript{6} unirradiated MC1A-C1 cells and 1.5 x 10\textsuperscript{7} irradiated (0.5-5 Gy) MC1A-C1 cells. In this experiment, the inducible level of 6-TG\textsuperscript{R} colonies in MC-TGS17-51 cells (calculated from linear regression analysis, with r = 0.959) was 203 per 10\textsuperscript{6} cells per Gy, or about 400 per 10\textsuperscript{6} viable cells per Gy. At 5 Gy, the estimated induced number of 6-TG\textsuperscript{R} colonies in the derived clone was at least 1000-fold greater than in the parental MC1A-C1 cell line. The sensitivity to cell killing by ionizing radiation was similar for the two cell lines, with a D\textsubscript{37} of 3.5 Gy for MC-TGS17-51 and 2.8 Gy for MC1A-C1. (D\textsubscript{37} was calculated from a linear quadratic curve fit to the data of Figure III.9 which gave r = 0.999 and 0.996 respectively)

3.4.3.2 Karyotyping. Karyotype analysis, using the X-specific hybridization probes of Breneman et al. (1993), was performed on the derived mouse fibrosarcoma cell lines. All three cell lines (MC1A-C1, MC-TGR17, and MC-TGS17-51) were found to be hypotetraploid with approximately three X-chromosomes and other smaller chromosome regions hybridizing to the fluorescent X-specific probes. Summary of karyotype analysis data is presented in Appendix 2.

3.4.3.3 Stability of 6-TG\textsuperscript{R} Mutants. MC-TGR17 cells, which had not been passaged through the mouse, demonstrated no revertants to the HAT\textsuperscript{R} phenotype in 10\textsuperscript{6} cells tested (plating efficiency 66%) after multiple passaging in vitro. However, the same clone, passaged through the mouse, demonstrated a HAT\textsuperscript{R} mutation frequency of 132 ±
Figure III.8  Generation of 6-TG$^R$ mutants by Co$^{60}$ γ-rays in MC1A-C1 and MC-TGS17-51 derived cell lines

▲, MC1A-C1 parental cell line; ●, MC-TGS17-51 derived cell line. Error bars represent the standard error of the mean, uncorrected for viability, of the average of 3-5 experiments, each involving 3-10 replicate plates.
Figure III.9  \( \text{Co}^{60} \gamma \text{ irradiation survival curves of MC1A-C1 and MC-TGS17-51 derived cell lines} \)

\( \Delta \), MC1A-C1 parental cell line; \( \bullet \), MC-TGS17-51 derived cell line. Error bars represent the standard error of the mean of the average of 3 and 8 experiments respectively, each involving 3-6 replicate plates.
21 revertants per $10^5$ viable cells after seven weeks of non-selective culturing (in vivo plus in vitro). The determination of spontaneous mutation frequency for this same clone following 1 week in non-selective medium yielded $26 \pm 4$ revertants per $10^5$ viable cells.

3.4.3.4 Origin of the Functional hprt Gene. The first evidence that the selected HAT$^R$ clones (MC-TGS17-1 to 54) may have been revertants of the endogenous mouse hprt gene, rather than successful transfectants, came from Southern analysis which detected no unique bands from the three generations of clones that were probed with the human pHPT37-D2 hprt minigene fragment (data not shown). Screening of the derived mouse fibrosarcoma cell lines for the transfected human hprt minigene, using PCR, failed to detect its presence in any of the DNA extracts tested despite the fact that the appropriate sequence was amplified in the pHPT37-D2 plasmid (Figure. III.10). The primers used in this PCR reaction would yield a 1633 bp product from a human endogenous or human minigene and a 950 bp product from a mouse endogenous gene, due to intron size variation (see Figure. III.11). Only the positive control demonstrated a 1633 bp band on the agarose gel following amplification, while the 950 bp fragment was seen in all cases. A separate control experiment (Figure III.12) demonstrated that even pg concentrations of the plasmid could be detected by this technique. The human specific reverse primer from intron 8 and the mouse/human reverse primer from exon 9 (Figure III.11), were also unable to amplify the human hprt minigene fragment in the test DNA but these primers were biased in amplifying the human (smaller 633 bp) fragment in the positive control (Figure III.13).

In 2 separate experiments, PCR screening of another 12 HAT$^R$ clones also failed
Figure III.10  PCR amplification of the mouse endogenous hprt gene or the human hprt minigene within the pHPT37-D2 plasmid

200 ng of eucaryotic DNA or 1 ng of plasmid DNA was used as the template for the PCR reaction. Exon 8 to exon 9 primers, capable of differentiating the murine and the human hprt genes, were employed to amplify the 950 bp and the 1633 bp fragments respectively. DNA extracted from the 4 generations of mouse cell lines (lanes 1-6), amplified a 950 bp band. Plasmid DNA, alone (lane 7), or in presence of the mouse DNA (lane 8), amplified a human specific 1633 bp fragment.

Figure III.11  PCR primers for amplification of the human endogenous hprt gene, the human hprt minigene and the mouse endogenous hprt gene

Exon 8 froward and exon 9 reverse primers, capable of differentiating the murine and the human hprt genes based on intron size sequences, were employed to amplify the 950 bp and the 1633 bp fragments respectively. A human specific intron 8 reverse primer together with exon 8 forward primer amplified a 950 bp human hprt gene or minigene fragment.
Figure III.10

MINIGENE AND HUMAN ENDOGENOUS hprt

EXON 8 1633 bp EXON 9

633 bp

MOUSE ENDOGENOUS hprt GENE

EXON 8 950 bp EXON 9

Figure III.11
Figure III.12  Evaluation of PCR amplification sensitivity

The primers used in this amplification reaction were designed to span exon 8 to exon 9 of both human and mouse *hpert* genes (same as in Figure III.11). Lanes 1-6 represent the PCR amplified products in the presence of 200 ng of mouse DNA with decreasing concentrations of pHPT37-D2 plasmid DNA: lane 1, 0 ng; lane 2, 1.0 ng; lane 3, 0.1 ng; lane 4, 10.0 pg; lane 5, 1.0 pg; lane 6, 0.1 pg. Lane 7 was the negative control, void of DNA. Lanes 8-12 represent the PCR amplified products in the presence of increasing concentrations of pHPT37-D2 plasmid DNA: lane 8, 0.1 pg; lane 9, 1.0 pg; lane 10, 10.0 pg; lane 11, 0.1 ng; lane 12, 1.0 ng. Marker (lane 13), was a *Hind* III digest of pCB10 plasmid.

Figure III.13  Three primer PCR amplification of the mouse endogenous *hpert* gene or the human *hpert* minigene within the pHPT37-D2 plasmid

200 ng of eucaryotic DNA or 0.1 ng of plasmid DNA was used as the template for the PCR reactions. All lanes, except 13, represent the amplified products of a reaction containing universal (mouse and human) primers exon 8 to exon 9, as well as an intron 8 human specific reverse primer. Lane 13 represents the amplification product of exon 8 to intron 8. DNA templates of each lane: 1, MC1A-C1; 2-4, MC-TGR-17; 5, MC-TGS17-51; 6, MN-11; 7-10, 6-TGR spontaneous mutants of MN-11; 11, pHPT37-D2; 12-13, MC1A-C1+ pHPT37-D2; 14, no DNA; 15, *Hind* III pCB10 marker.
Figure III.12

Figure III.13
to demonstrate the presence of the transgene (Figures III.14 and III.15). Finally, concluding evidence came from RT-PCR analysis of MN-11 cells, a clone derived from MC-TGS17-51, where only a mouse specific 289 bp band was detected. A human specific RNA of 310 bp was only present in human fibroblasts and in the positive control mix of MC1A-C1 and human fibroblast cells (Figure III.16).

3.4.4 SUMMARY

The difficulty in inducing hprt mutants in the MC1A-C1 cell line was demonstrated by testing more than 1.8 x 10^7 cells for both spontaneous and radiation induced loss of gene function. This difficulty contrasted with the observed ease of detecting mutants in the derived clone MC-TGS17-51. These observations gave more confidence to the suggestion that multiple functional hprt genes were present in MC1A-C1 and that all had to be inactivated to result in selection of a 6-TG^R mutant. Karyotypic analysis of the three generations of mouse fibrosarcoma cell lines (MC1A-C1, MC-TGR17 and MC-TGS17-51) supports the theory of multiple hprt genes by demonstrating the presence of 3 X-chromosomes and X-specific fragments on other chromosomes.

The successful selection of 6-TG^R mutants (section 3.2) implies that all endogenous hprt gene(s) were inactivated. Subsequent electroporation and recovery of HAT^R colonies was originally believed to be the result of a successful transfection with the human hprt mini-gene. However, the high spontaneous reversion frequency to HAT resistance prior to electroporation, observed in the MC-TGR17 clone, opened up the possibility that some of the MC-TGS17 clones were actually “activating” revertants of
Figure III.14  Three primer PCR amplification of the mouse endogenous \textit{hprt} gene in different MC-TG\textsuperscript{s} clones -- "No evidence of human \textit{hprt} minigene in several MC-TG\textsuperscript{s} clones."

200 ng of eucaryotic DNA or 0.1 ng of plasmid DNA was used as the template for the PCR reactions. All lanes represent the amplified products of a reaction containing universal (mouse and human) primers exon 8 to exon 9, as well as an intron 8 human specific reverse primer. DNA templates in each lane: 1, MC-TGS17-1; 2, MC-TGS17-19; 3, MC-TGS17-20; 4, MC-TGS17-21; 5, MC-TGS17-25; 6, MC-TGS17-28; 7, MC-TGS17-36; 8, MC-TGS17-43; 9, MC-TGS17-49; 10, MC-TGS17-50; 11, pHPT37-D2; 12, no DNA; 13, \textit{HindIII} pCB10 marker.

Figure III.15  Three different PCR amplifications of the mouse endogenous \textit{hprt} gene and the human endogenous gene or \textit{hprt} minigene

Representation of three separate PCR experiments (lanes 1-9), each using 200 ng of eucaryotic DNA or 0.1 ng of plasmid DNA. Each group primed with different set of primers: 1, exon 8-9; 2, exon 8 to human intron 8; 3, exon 8-9 + human intron 8 reverse primer. DNA templates in each lane: 1, no DNA; 2, MC1A-C1; 3, MC-TGS17-17; 4, MC-TGS17-39; 5, human TK6 lymphoblastoid cell line; 6, induced \textit{hprt} mutant of TK6; 7, TK6 \textit{hprt} mutant transfected with \textit{hprt} minigene; 8, human white blood cells; 9, pHPT37-D2; M, \textit{HindIII} pCB10 marker.
Figure III.16  Reverse transcriptase PCR amplifications of the *hprt* RNA isolated from the mouse and the human cells

RNA, reverse transcribed and PCR amplified using exon 1 forward primers, specific for human or mouse *hprt* gene, and a universal exon 3 reverse primer. Templates for PCR reaction: 1, human fibroblast; 2, MC1A-C1; 3, MC-TGR17; 4, MN-11; 5, human fibroblast + MC1A-C1; M, 100 bp ladder marker.
Figure III.16
the different endogenous hprt genes. The observation that passaging of the 6-TG\textsuperscript{R} mutant clone through the mouse appeared to induce this phenotypic switching from 6-TG\textsuperscript{R} to HAT\textsuperscript{R} phenotype, without the presence of the transgene, may be suggestive of the in vivo environment contributing in some fashion, yet unknown, to genomic instability.

The possibility that hprt marker gene(s) were relocated through recombinational events to different sites on the chromosomes, creating targets for clastogenic agents of different size, may also explain the observed hprt gene expression differences in the clones tested. The outcome of an exposure to a clastogenic agent would depend on the probability of a target site receiving an inactivating "hit". For different targets at different chromosomal locations, the “hit” probability would depend on the size of each target. Under these conditions, each specific location of the gene would be associated with slightly different target size, thus a different sensitivity of detecting both spontaneous and induced mutational events. However, for MC-TGS17-51 to detect induced mutations at the observed frequencies, equivalent to other heterozygous gene markers, suggests that the single functional hprt gene was on a heterozygous chromosome.

Strong evidence for the reactivation of a previously inactivated endogenous mouse hprt gene, rather than a successful transfection, comes from Southern analysis, PCR and RT-PCR data. Under all circumstances tested, there was no evidence of the human transgene in any of the “transfected” clones tested. The suggestion that some of the most mutable clones may have an unstable incorporation of the hprt transgene was also discounted since no human specific bands appeared in any of the reactions. Thus, the selected clone, and possibly all HAT recovered mutants, were a consequence of
manipulation and phenotypic selection of endogenously occurring events which lead to a 
selectable marker present on a heterozygous chromosome, suitable for use in sensitive 
detection of mutations. In light of the findings presented in Chapter 4, this serendipitous 
event may have created a more favourable model system than anticipated at the outset of 
this project.
3.5 DEVELOPMENT OF AN *IN VIVO* MOUSE MUTAGENESIS MODEL

3.5.1 OBJECTIVES

The primary use of the developed experimental model was to determine if the frequency of 6-TG$^R$ mutants in implanted subcutaneous tumours was affected by the *in vivo* environment in syngeneic mice. Our hypothesis states that factors within the tumour environment are mutagenic and potentially able to enhance the process of tumour progression. In support of our hypothesis, a recent publication (Paquette and Little, 1994) demonstrated the enhanced genomic instability of mouse transformed cells *in vivo* over that observed *in vitro*.

Transgenic animals have also been used for monitoring mutations within the tumour environment (Hundley et al., 1997; Jakubczak et al., 1996), however these studies have limitations because of the high spontaneous background mutations at the marker gene decreasing the sensitivity of the assay (Skopek et al., 1995; Gossen et al., 1991). Furthermore, studies using shuttle vectors are designed for detecting mutations within the gene of interest, and therefore underestimate large scale chromosomal mutations (Douglas et al., 1994).

In this thesis a transplantable tumour cell line (MC-TGS17-51) with increased sensitivity for detecting mutational events (both genomic and chromosomal) could then be used to more completely test the hypothesis and compare the *in vivo* mutation
frequencies with those acquired under in vitro conditions for the same period of time.

Four other cell lines, demonstrating different spontaneous and induced mutation frequencies were also assessed for their ability to detect in vivo mutational events.

3.5.2 METHODOLOGY

3.5.2.1 In Vivo Mutation Frequencies. Five MC-TGS17 clones were screened for hprt expression by culturing in HAT medium for 7 days to remove pre-existing 6-TG<sup>R</sup> mutants. Following this "purification", 6 syngeneic C57BL/6 mice per clone were subcutaneously injected with 10<sup>6</sup> cells in 0.1 ml of PBS and the subsequent mutation frequencies were established for cells recovered from excised tumours after 15 to 21 days of growth (as described in section 2.2.5). The mutation frequencies found in these cells were compared to the mutation frequencies found in equivalent cell lines cultured under in vitro conditions for the same period of time.

3.5.3 RESULTS

3.5.3.1 In Vivo Mutation Frequencies. For each clone, the mean number of 6-TG<sup>R</sup> mutants recovered from 4 to 6 tumours were compared to the mean numbers of spontaneous 6-TG<sup>R</sup> mutants recovered after an equivalent time of culture in vitro (Fig. III.17). It was observed that 3 out of the 5 clones tested demonstrated an increase in mutation frequency in cells recovered from tumours when compared to those grown in vitro, with clone MC-TGS17-51 being one of the three. However, clones MC-TGS17-1 and MC-TGS17-28 demonstrate a decreased accumulation of 6-TG<sup>R</sup> mutants during in
Detection of 6-TG$^R$ mutants arising in vivo in different MC-TG$^S$ tumours

represent the mutation frequency of cells grown in vitro under non-selective conditions for 14-25 days before being challenged with 6-TG. The mean ± standard error of the mean of 4-6 replicate dishes in a single experiment is shown. represents the mean mutation frequency of cells recovered from different MC-TG$^S$ tumours following equal period in vivo incubation. The error bars indicate the standard error of the mean: four tumours for clones MC-TGS17-1 and MC-TGS17-51; five tumours for clones MC-TGS17-25 and MC-TGS17-36, and six tumours for clone MC-TGS17-28. The in vitro and in vivo mutation frequencies data were compared for each clone using a nonparametric test (Mann-Whitney); MC-TGS17-1, P=0.0286 considered significant; MC-TGS17-25, P=0.0317 considered significant; MC-TGS17-28, P=0.0087 considered very significant, MC-TGS17-36, considered very significant, and MC-TGS17-51, considered not significant.
vivo growth. The *in vitro* and *in vivo* mutation frequencies data were compared in a nonparametric test (Mann-Whitney test), assuming non-Gaussian distribution of unpaired columns, and the two-tailed P values are reported in the figure legend III.17. It is important to note that large variation in mutation frequencies was observed between the animals even within the same test population, and thus for statistical significance, a greater number of tumours had to be examined for clone MC-TGS17-51. A more extensive comparison of *in vivo* and *in vitro* mutation frequencies was conducted by J.K.S. Sandhu using the MN-11 cell line, a clonal derivative of MC-TGS17-51. These results demonstrated that the mutation rates obtained *in vivo* were increased 3.4 fold over those obtained from cells cultured *in vitro* (Wilkinson et al., 1995).

3.5.4 SUMMARY

Our hypothesis states that factors within the tumour environment may be contributing to mutation frequency and thus to the process of tumour progression. It is now well accepted that spontaneous mutation frequencies must be accelerated in the tumour environment to account for the large number of mutations found in an individual tumour (Cheng and Loeb, 1997). Work by Paquette and Little, (1994) supports this theory by demonstrating an enhanced genomic instability in the minisatellite sequences of cells grown in syngeneic animals when compared to that observed in cells cultured *in vitro* for the same period of time. However, the limitation of their model is the need for a 6-week *in vitro* culture period of tumour cells for the purpose of eliminating invading normal cells prior to detection of mutants in the tumour cells. It is possible that clonal
selection during this in vitro culture period may be affecting genomic instability of the recovered clones.

Our own work further demonstrates that the tumour environment is contributing to tumour cell instability, leading to tumour cell heterogeneity which, in turn, is known to enhance tumour progression (Nowell, 1982). Large variance in mutant frequency among different tumours compared to small variance in replicates of a single tumour cell suspensions demonstrated in these experiments suggests that each tumour was unique in accumulating mutational events, possibly due to the probabilities involved in induction and growth selection of each induced mutant cell as a consequence of tumour growth heterogeneity, as discussed by Kendal and Frost (1988). This would explain how a cell acquiring a mutation early in the tumour development may be underrepresented in final cell suspension possibly due to simultaneous mutations leading to loss of viability, or tumour physiology resulting in regional necrosis.

Preliminary data in figure III.17 suggest that certain clones exhibited an increased number of mutational events following tumour excision while two demonstrated a decrease during in vivo growth. The observed decrease in mutation frequencies may be explained by an in vivo induced selection pressure acting against the 6-TG R mutants, a slower growth rate of some mutants, or a balance between mutation and in vivo reactivation of a previously inactive endogenous hprt gene. Two clones demonstrated a statistically significant enhancement of in vivo mutation induction, possibly attributable to the tumour environment. Clone MC-TGS17-51 demonstrated no significant difference between the in vitro and the in vivo sets of data in this experiment. In a more extensive
study, the number of mutational events detected in the tumour was increased 3 to 4 fold over the number found in the same MC-TGS17-51 cells cultured in-vitro, an enhancement that was attributed to the more mutagenic in vivo environment (Wilkinson et al., 1995).

In conclusion, genomic instability appeared to be enhanced in the in vivo environment in at least some of the tumours studied. This observation supports our hypothesis that there are factors present in the tumour environment that are contributing to genomic instability.
3.6 SECTION DISCUSSION

Work by Lichtenauer-Kaligis (Lichtenauer-Kaligis et al., 1995) demonstrated that UV-induced mutation frequencies, while 7.5 fold higher than observed at the endogenous \textit{hprt} gene locus, were similar for the three cell lines expressing HPRT from cDNA retroviral vector sequences integrated into different genomic positions. In contrast to U.V. damage, which was associated with small scale mutational events or intragenic events (Peak and Peak, 1988; Turker et al., 1995), clastogenic agents such as Co\textsuperscript{60} γ-rays are known to induce large scale mutational events. These large scale mutational events would be expected to result in differences in mutation frequencies observed in cells where the marker gene, such as the \textit{hprt} gene, was present in different chromosomal locations.

Genetic manipulation and clonal selection led to the isolation of a number of clones with different sensitivities of detecting mutational events at the endogenous \textit{hprt} gene locus. The most stable clone (MC-TGS17-51) demonstrated a rate of mutation induction at the \textit{hprt} locus to be 27 fold greater than that previously reported for the endogenous X-linked \textit{hprt} in the mouse cell line (Evans et al., 1986b). However, the observed mutation frequency in the experiments reported here was within the range reported for other heterozygous marker genes (\textit{tk, aprt} and \textit{gpt}) in mouse and Chinese hamster lines (Evans et al., 1986b; Bradley et al., 1988; Ikehata et al., 1989; Schwartz et al., 1991). These data strongly support the argument that the selected clone, MC-TGS17-51, had a single functional \textit{hprt} marker gene located on a heterozygous chromosome. This cell line has the capacity to be used as a sensitive detector of mutational events in
vitro, and furthermore, it was tested for its ability to detect mutational events within an in vivo environment.

However, the observation that different HAT$^R$ clones appeared to demonstrate different spontaneous and induced mutation frequencies supports the possibility that endogenous hprt marker genes, located at different sites within the genome, may be differentially affected by clastogenic events, further emphasizing the importance of marker gene location in detecting mutational events, even under heterozygous conditions. This cell line was originally isolated from a male mouse, where a normal cell would only have a single copy of the X-chromosome. Presence of multiple X-chromosomes in the tumour cell suggests inappropriate segregation of replicated X-chromosome, each potentially expressing the endogenous hprt gene. Work by Takagi (1993) demonstrated a variation in inactivation pattern of the X-chromosomes in tetraploid murine embryonal carcinoma fused with lymphocyte somatic cells. This work suggests that the X-inactivation mechanism operating in diploid cells may not be correctly functional in polyploid cells, giving rise to various inactivation patterns in tetraploid cells. The irregular X-inactivation may induce chromosomal variation leading to maintenance of three functionally unique X-chromosomes. This irregularity would explain the observed differences in the spontaneous and induced mutation frequencies of the hprt gene in the different HAT$^R$ clones examined. At least two, possibly three or more, hprt genes were originally present in the MC1A-C1 cell line. All functional genes would have to be mutated to result in an HAT$^R$ phenotype. Assuming that the three X-chromosomes were identical, the probability of multiple gene inactivations occurring in a single cell can be
approximated by the induced mutation frequency event at the locus of a single heterozygous \textit{hpRT} gene marker raised to the power of the number of different chromosomal locations of the gene, or more precisely, the last gene to be mutated would accumulate mutations at a rate similar to that of a hemizygous marker. For 2 independent \textit{hpRT} genes (i.e. on 2 separate chromosomes) the probability of mutating both in a single cell using 1 Gy of ionizing radiation would be approximately equal to the product of mutation frequency at a heterozygous marker times the mutation frequency at a hemizygous marker \((400 \times 10^{-6})(15 \times 10^{-6}) = 6 \times 10^{-9}\). For 3 \textit{hpRT} genes this number would be \((400 \times 10^{-6})(400 \times 10^{-6})(15 \times 10^{-6}) = 2.4 \times 10^{-12}\). In these calculations it is assumed that the heterozygous mutation frequency was similar to that observed at the MC-TGS17-51 \textit{hpRT} locus, from Table III.4 data, adjusted for 50\% plating efficiency, and the hemizygous mutation frequency was similar to that reported by Evans et al. (1986b). The maintenance of three X-chromosomes throughout the three derived sub-clones over the course of 8 years of culturing, further suggests that the 3 X-chromosomes may be non-identical in that each contains some essential genes not found in the other two. Recent work by Dr. D. Blakey (unpublished information) using mouse X-specific centromeric probes demonstrates that all three chromosomes are maintained in MC-TGS17-51 6-TG\textsuperscript{R} mutants suggesting that loss of the whole chromosome is not a likely event. This implies that all 3 X-chromosomes were unique in preserving different essential genes, possibly due to mutagenic conditions used to initiate the tumour or enhancement of homologous recombination (spontaneous or induced by DNA damage) facilitated by nuclear segregation of the three X-chromosomes. Moreover, X-
chromosome differentiation may have been introduced by inactivation of repetitive regions by DNA methylation and subsequent G-C to A-T mutation, a suggested mechanism of introducing diversity into repetitive gene sized regions longer than 0.3 kb (section 1.5.1 page 19; section 4.5.1; Kricker et al., 1992). It is also possible that MNU, acting as a recombinogenic agent (Ramel et al., 1996), had enhanced diversity between the three X-chromosomes; however, this would only intensify the already existing differences between the 3 stably maintained chromosomes prior to this treatment. Figure III.18 presents a possible model for preservation of 3 distinct X-chromosomes.

In conclusion, this chapter described the development of a number of cell lines, of which one was capable of sensitive detection of presumed small scale and large scale mutational events under in vitro and in vivo conditions. For this derived cell line, the experimental evidence suggests the genomic marker was located on a heterozygous chromosome (most likely the X-chromosome) at a site that resulted in a large target size for the detection of mutational events.
Figure III.18  Model for evolution of a mouse fibrosarcoma cell line with 3 unique X-chromosomes

A single functional X-chromosome in a normal mouse cell becomes replicated to produce 3 identical X-chromosomes through a process of non-disjunction. Early mutagenesis by spontaneous or methylcholanthrene induced events of MC1A cell line possibly resulted in diversification of the 3 X-chromosomes, where all three are essential for viability. □ Represents a normal functional gene. ● Represents a mutant or inactive gene. ○ Represents a gene that is unique in expression due to lack of expression of its homologues.
Non-disjunction

Early MC1A

Normal male mouse cell
(Single X-chromosome)

Methylcholanthrene induced fibrosarcome
(polyplloid with 3 identical X-chromosomes)

Early
Mutagenesis
or
Recombination

MC1A
3 Unique X-chromosomes

Figure III.17
CHAPTER 4

GENOMIC INSTABILITY OF ENDOGENOUS AND TRANSFECTED \textit{hprt} GENE IN A HUMAN LYMPHOBLASTOID CELL LINE

4.1 INTRODUCTION

In developing the transplantable mouse fibrosarcoma cell line described in Chapter 3, it was observed that some of the clones expressing the endogenous \textit{hprt} marker gene demonstrated variation in spontaneous and induced mutation frequencies. Based on these observations, it was postulated that marker gene location might be one factor affecting the ability of the assay to detect viable/scorable mutations. Moreover, the susceptibility of a gene to undergo mutations is known to be determined, in part, by its position within the genome, suggesting that genomic positioning could also affect genomic stability/instability.

It is the hypothesis of this thesis that the probability of a marker gene being lost or inactivated by spontaneous and induced mutational events is highly dependent upon its chromosomal location. To explore this hypothesis, the strategy was to inactivate an endogenous selectable gene (\textit{hprt}), ideally by deletion, and then randomly insert into the genome a \textit{hprt} minigene as a marker of mutational events. To be optimal as a genetic
marker, it was important to transfect in sequences that would not be considered “foreign” to the host cell, that is, avoiding bacterial or viral sequences. A human cell line was selected to exploit the use of procedures because of its relative ease compared to murine cells for karyotypic analysis and fluorescent in situ hybridization and because of the advanced state of understanding of the human genome.

The intent was to select a number of transfectants where the minigene had integrated into different regions on the chromosome. It was hoped that total deletion of the endogenous hprt gene would prevent biased integration of the minigene. These transfectants could then be monitored for stability of transgene expression under spontaneous or induced conditions. An assumption was made that transgene integrations would be random; however, as previously stated, there is evidence that chromosomal fragile sites pre-existing or arising as a consequence of electroporation, could be preferential sites of insertion for invading double stranded DNA fragments (Kato et al., 1986; Rassool et al., 1991).

Numerous studies performed with transgenic cell lines indicate that the stability of the transgene expression could be highly variable (Krumlauf et al., 1986; Pravtcheva et al., 1994; Kearns et al., 1995; Wallrath and Elgin, 1995; Robertson et al., 1995). Despite this evidence, the reasons for this gene expression variegation are, as yet, unknown. The results of this study could further clarify the fate of transgenes in host cells. This knowledge could be useful for human gene therapy by providing guidance in optimizing the maintenance of expression of transgenes within the host.
4.2 CELL LINE SELECTION AND MUTATION
OF THE ENDOGENOUS *hprt* GENE

4.2.1 BACKGROUND INFORMATION

To ensure the objectives of this study could be met, a suitable cell line had to be chosen that was derived from a male donor and had an apparently normal karyotype. Since *Hprt* was chosen to be the transfected marker gene, complete deletion of the endogenous *hprt* gene was necessary to avoid reversion and interference with transgene integration. Several human cell lines were screened for suitability: i) LS-174T colon cancer cell line proved to be too mucoid and difficult to desegregate into a single cell suspension. ii) IMR-32 human neuroblastoma cell line had an inordinately long doubling time of ≥ 100 h. iii) A primary human fibroblast from a newborn male (supplied by Dr. R. Korneluck, Children's Hospital of Eastern Ontario, Canada) demonstrated sensitivity to 6-TG but displayed very low plating efficiency, approximately 3%. iv) A primary human fibroblast from a normal newborn male (supplied by Dr. S. Bennett, Ottawa Regional Cancer Centre, Canada) failed to demonstrate sensitivity to the toxic effects of 6-TG. In a similar experiment where drug resistance was observed (Swann et al., 1996), it was suggested that cells lacking a component of the postreplicative mismatch repair pathway may display resistance to this cytotoxic drug.

The TK6 cell line was eventually chosen because it has a stable diploid karyotype (Yandell and Little, 1986), and was isolated from a male donor (Liber and Thilly, 1982).
It has a single X-chromosome, a short doubling time, an endogenous heterozygous \textit{tk} gene on chromosome 17 (providing an endogenous marker of genomic instability (Thilly et al., 1980; Liber et al., 1989; Giver and Nelson, 1995)), and it could grow in suspension culture allowing more cells to be tested per tissue culture plate. A methylcellulose-RPMI 1640 medium was utilized to detect clonal events within a population of TK6 cells in suspension (Buick et al., 1979). For a more detailed summary and description of TK6 and derived clones see Appendix I.

The first objective in the experimental strategy was to eliminate the endogenous \textit{hprt} gene by selecting a deletion mutant with low probability of spontaneous reversion to \textit{HAT}\textsuperscript{R} phenotype. Different clastogenic agents were used to induce DNA strand breaks and recover 6-TG\textsuperscript{R} phenotype mutants. Bleomycin-iron (III) citrate (Burger et al., 1995), a radiomimetic drug (Povirk, 1996), was expected to induce mutations by producing DNA double strand breaks with subsequent deletions of the \textit{hprt} gene since Köberle and Speit, (1991) showed that 43% of the bleomycin induced mutants had a partial or total deletion of the \textit{hprt} coding sequences. Characterization of bleomycin mutants as well as mutants recovered after Co\textsuperscript{60}\gamma irradiation led to a selection of an 6-TG\textsuperscript{R} clone that could be transfected with the human \textit{hprt} minigene.

The possibility that some of the selected 6-TG\textsuperscript{R} mutants arising from these treatments have been due to methylation-induced inactivation was tested. It has been previously reported that the endogenous \textit{hprt} gene could be inactivated by hypermethylation (Lock et al., 1986; Grant and Worton, 1989), and that continuous maintenance of cells in tissue culture could disrupt normal methylation patterns.
(Schorderet et al., 1988). Randomly selected mutants were therefore tested for reversion by exposure to 5-azacytidine, a DNA methylation inhibitor shown to be capable of reactivating a gene silenced by DNA methylation (Holliday and Ho, 1995).

6-TG<sup>R</sup> mutant clones, that could not be phenotypically reversed by 5-azacytidine, were screened for deletion of the <i>hprt</i> gene by PCR analysis. Those that demonstrated lack of PCR amplification product were further characterized for doubling times, radiation sensitivity, spontaneous reversion to HAT<sup>R</sup> phenotype and spontaneous mutation frequency at the endogenous <i>tk</i> gene locus.

4.2.2 METHODOLOGY

4.2.2.1 Clonogenic assay for lymphoblastoid cells. The use of methylcellulose-RPMI 1640 culture medium proved to be the method of choice for detecting mutants by a clonogenic assay for anchorage independent TK6 cells. Culture conditions for non-selective and selective growth of cells and mutants were described in section 2.2.

4.2.2.2 Characterization of TK6 cell line. To confirm earlier karyotypic analysis (Yandell and Little, 1986), metaphase spreads of TK6 cells were prepared for cytogenetic analysis, as described in section 2.6.1. The doubling time was determined by performing repeated cell counts on a fraction of a population in at least three separate plates, over a period of time during which the cells display logarithmic growth (described in 2.2.3).

4.2.2.3 Induction of 6-TG<sup>R</sup> mutants. TK6 cells were treated with a number of different mutagens in an effort to recover a mutant lacking the endogenous <i>hprt</i> gene. Different doses of bleomycin-iron (III) citrate complex were used to induce mutations
(see section 2.4.3). At each dose, a fraction of cells was used to determine plating efficiency in non-selective medium. For isolation of 6-TG\textsuperscript{R} mutants, 2 to 6 plates were maintained in non-selective medium for 7 days prior to challenge with 6-TG (10\textsuperscript{5} cells per 96 multiwell plate). All plates were cultured and wells were scored for growth. Potential mutants were subcultured and further characterized.

4.2.2.4 Characterization of bleomycin induced mutants. The spontaneous and bleomycin induced mutants were randomly selected (each from a different treatment plate) and tested for phenotypic reversion following exposure to 5-azacytidine to determine if inactivation of the \textit{hprt} gene was due to hypermethylation. The concentration of 5-azacytidine to be used for phenotype reversion experiments was based upon an estimate of toxicity obtained from the growth curves of TK6 cells and from survival curve following 63 h treatment with various concentrations of 5-azacytidine. A total of 11 clones, 2 recovered from controls and 9 from bleomycin treated cultures, were treated with 1 \mu M 5-azacytidine for 63 hours, washed in PBS and then set-up to detect revertants in HAT and plating efficiency in non-selective medium. DNA from the above clones was subsequently subjected to PCR amplification to detect presence or absence of \textit{hprt} exon 8 to exon 9 sequences (as described in Figure III.11). The possible contamination of 6-TG\textsuperscript{R} clones with DNA from non-viable (6-TG\textsuperscript{S}) cells, encoding non-mutant \textit{hprt} was investigated. Fourteen mutants selected from bleomycin treated cultures were re-cloned and re-tested by PCR for presence of the endogenous \textit{hprt} gene. Clones which did not amplify a specific \textit{hprt} band were re-tested for the presence of DNA using the alternative internal standard, the thymidylate synthase (\textit{ts}) gene.
4.2.2.5 Co\textsuperscript{60} \(\gamma\)-ray induced mutants. Co\textsuperscript{60} \(\gamma\)-rays were employed in an attempt to recover 6-TG\textsuperscript{R} large scale deletion mutants at the \(hprt\) gene locus. Survival curves were established (described in section 2.4.1) and 11 mutants with the required phenotype were recovered from independent cultures for further characterization and selection. Mutants were first tested for the presence of \(hprt\) genomic sequences by using PCR to amplify two different (a 5' and a 3') regions of the endogenous gene. Assessment of doubling times, spontaneous reversion frequency from 6-TG\textsuperscript{R} to HAT\textsuperscript{R} phenotype, spontaneous mutation frequency at the \(tk\) locus detected by TFT challenge, and radiation sensitivity differences were used as criteria to select the appropriate clone for the transfection experiment (described in section 2.3.2).

4.2.3 RESULTS

4.2.3.1 Plating efficiency assay for lymphoblastoid cells. In most experiments, the plating efficiency in methylcellulose-RPMI 1640 was as good or better than scoring for growth using RPMI 1640, in 96 well plates. Mean plating efficiency in methylcellulose-RPMI 1640 of 103 data points was 49\% \pm 15\% S.E.M., (range from 20 to 80 \%), as compared to 20-50\% in 96 well plates with RPMI 1640 medium (data presented per each individual experiment). Individual 6-TG\textsuperscript{R} or HAT\textsuperscript{R} colonies were generally easy to score, with only occasional blurring between colonies (Figure IV.1).

4.2.3.2 Characterization of TK6 cell line. Preliminary karyotype analysis of the TK6 cell line demonstrates a male cell line with possible translocations and trisomy 13 where \(2N = 46\) (Figure IV.2). Doubling time for TK6 cells was estimated to be between
Figure IV.1  Clonogenic assay for TK6 lymphoblastoid cells

TK6 cells, normally cultured in suspension were grown in 0.8% methylcellulose-RPMI 1640 culture medium. 1a) typical appearance of easily scorible colonies. 1b) Occasional blurring of colonies causing difficulty in scoring.

Figure IV.2  Metaphase spread of a TK6 lymphoblastoid cell

A metaphase spread of a typical TK6 cell demonstrating a stable karyotype where 2N=46.
13.7 and 14.6 hours calculated from 4 independent experiments with a mean value of 14 ± 0.4 h (growth curves shown later in Figure IV.15).

4.2.3.3 Characterization of bleomycin induced mutants. Initial treatment of TK6 cells with various concentrations of bleomycin-iron (III) citrate complex resulted in the recovery of 89 6-TG<sup>R</sup> mutants (spontaneous and induced), from 19 plates, where each plate was seeded with 10<sup>5</sup> cells. Observed plating efficiencies and mutation frequencies corrected for viability were reported for each dose tested (Table IV.1).

Table IV.1 Plating efficiencies and mutation frequencies of bleomycin treated TK6 cells.

<table>
<thead>
<tr>
<th>Bleomycin Concentration</th>
<th>0 µM</th>
<th>1 µM</th>
<th>3 µM</th>
<th>10 µM</th>
<th>30 µM</th>
</tr>
</thead>
<tbody>
<tr>
<td># 6-TG&lt;sup&gt;R&lt;/sup&gt; Wells / # Plates Tested</td>
<td>8/2</td>
<td>42/6</td>
<td>23/6</td>
<td>11/3</td>
<td>5/2</td>
</tr>
<tr>
<td>Plating Efficiency</td>
<td>22 %</td>
<td>22 %</td>
<td>10.4 %</td>
<td>6.3 %</td>
<td>5.2 %</td>
</tr>
<tr>
<td>Mutation Frequency Corrected For Plating Efficiency</td>
<td>1.8 / 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>3.2 / 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>3.7 / 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>5.8 / 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>1.9 / 10&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

4.2.3.4 5-azacytidine challenge for phenotypic reversion at the hprr locus. The high number of spontaneously arising mutants in the above experiment suggested that they may be a consequence of reversible hprr gene inactivation possibly due to methylation. Preliminary data from Figure IV.3 (a) and (b), suggest that a 63 h treatment of TK6 cells with 1 µM 5-azacytidine results in approximately 50% reduction in the
Figure IV.3a  Survival of TK6 lymphoblastoid cells in the presence of 5-azacytidine (trypan blue exclusion assay)

TK6 cells, maintained in the presence of different concentrations of 5-azacytidine were monitored for viability by trypan blue exclusion assay. Data points represent a mean number of viable cells sampled from at least 3 different cultures for each dose tested (±S.E.M.).

Figure IV.3b  Survival of TK6 lymphoblastoid cells after a 63 hour treatment with different concentrations of 5-azacytidine (plating efficiency assay)

TK6 cells, treated with different concentrations of 5-azacytidine were monitored for survival by the plating efficiency assay. Data points at each dose represent the fraction of treated clonable cells to untreated controls from at least 3 different culture plates scored after an 11 to 14 day incubation period (±S.E.M.).
number of viable cells when compared to the controls, and ≤ 20% decrease in survival following treatment, a strategy that was used in future experiments. Eleven 6-TG<sup>R</sup> mutants were challenged with 5-azacytidine to monitor for the possibility of reversion to the HAT<sup>R</sup> phenotype. A single clone, one of two spontaneously arising mutants tested, demonstrated phenotype reversion to HAT<sup>R</sup> after treatment with 5-azacytidine or after no treatment (reversion frequencies of 26 ± 2.8% S.E.M. and 32 ± 5.5% S.E.M. respectively, both values were corrected for viability). However, the same clone survived a normally lethal challenge with aminopterin alone, indicating that HAT<sup>R</sup> phenotype was a consequence of aminopterin resistance rather than reactivation of the hprt gene. The remaining 10 clones, 1 spontaneous and 9 from bleomycin treated cultures, were unresponsive to spontaneous or 5-azacytidine induced reversion to HAT<sup>R</sup> phenotype. Each clone was tested and no revertants were detected in a total of approximately 5 × 10<sup>5</sup> clonable untreated or 10<sup>3</sup> to 10<sup>4</sup> clonable 5-azacytidine treated cells. The plating efficiencies for the two parallel experiments ranged between 28 - 48% in the non-treated clones and between 1 - 13.5% in the 5-azacytidine treated equivalents.

**4.2.3.5 Characterization of bleomycin induced mutants by PCR.** The 11 6-TG<sup>R</sup> clones (discussed in section 4.2.3.4) demonstrating the appropriate phenotype, were further screened by PCR to select one that was a deletion mutant lacking the entire hprt gene. However, PCR of the genomic sequences spanning intron 8 produced the expected hprt gene product of 1633 bp (Figure IV.4). Re-cloning of 14 (6-TG<sup>R</sup>) bleomycin-induced mutants followed by PCR amplification, produced only 2 clones that did not appear to amplify the hprt fragment (Figure IV.5). However, these same two clones,
**Figure IV.4**  
No evidence of large scale mutations at the *hprt* locus in 11 bleomycin-induced or spontaneous mutants

6-TG^R^ mutants were tested for the presence of *hprt* (exon 8 - 9) by PCR analysis. A product of 1633 bp indicates the presence of the human specific fragment, as demonstrated in TK6, human lymphocyte and pHPT37-D2 DNA (lanes 1, 2, and 14 respectively). Mutant clones (lanes 3 and 4 spontaneous, and lanes 5 to 13 bleomycin-induced) amplify the same 1633 bp fragment. No bands were apparent in lane 15 containing no DNA. Restriction digested pCB10 was used as a molecular weight marker, lane M.

**Figure IV.5**  
Examining for evidence of large scale mutations at the *hprt* locus in 14 re-cloned bleomycin-induced mutants

Fourteen 6-TG^R^ bleomycin-induced mutants were re-cloned and tested for the presence of *hprt* genomic fragment (exon 8 - 9) by PCR analysis. A 1633 bp product indicates the presence of the human specific fragment, as demonstrated in TK6 DNA (lane 1). Of the 14 mutant clones tested (lanes 2 - 15), only the mutant in lane 5 shows no evidence of any bands while the one in lane 13 had a weaker band at 1633 bp. No bands were apparent in lane 16 containing no DNA. Restriction digested pCB10 was used as a molecular weight marker, lanes M.
when re-tested with an internal standard (ts) and failed to show a product (not shown).

4.2.3.6 Co\textsuperscript{60} γ-ray induced 6-TG\textsuperscript{R} mutants. Since bleomycin treatment did not produce desired clones with hprt gene deletion, mutation induction was repeated using Co\textsuperscript{60} γ-rays. To ensure recovery of mutants after irradiation, a kill curve was first established (Figure IV.6). Wild type TK6 cells, treated with CHAT to eliminate pre-existing hprt and tk mutants, were irradiated with 50-500 rads of Co\textsuperscript{60} γ-rays which led to isolation of 36 6-TG\textsuperscript{R} mutants (Table IV.2).

<table>
<thead>
<tr>
<th>Table IV.2</th>
<th>Induction of 6-TG\textsuperscript{R} mutants with Co\textsuperscript{60} γ-rays</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Co\textsuperscript{60} γ-rays (rads)</td>
</tr>
<tr>
<td>------------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>6-TG\textsuperscript{R} clones Recovered</td>
<td></td>
</tr>
<tr>
<td># Of Viable Cells Tested</td>
<td></td>
</tr>
<tr>
<td>Day 7 Mutation Frequency</td>
<td></td>
</tr>
</tbody>
</table>

4.2.3.7 Characterization of Co\textsuperscript{60} γ-ray induced mutants. The presence of a hprt-specific 1633 bp PCR fragment immediately eliminated 7 out of the 11 6-TG\textsuperscript{R} clones as potential deletion mutants (clones 1-7) (Figure IV.7). One clone amplified neither ts nor hprt gene, indicating poor template DNA. However, DNA from 3 clones were capable of amplifying the ts gene fragment (seen as two bands of 838 and 851 bp, after EcoRV digestion) without obvious amplification of the hprt gene fragment of 1633 bp (Fig.IV.8). These same clones were tested for possible amplification of the 5' region of the hprt gene, and once again no product was observed for the three mutants while a
Figure IV.6  **TK6 survival after Co\textsuperscript{60} γ-irradiation**

Radiation killing effects were monitored by establishing the surviving fraction of irradiated cells, using the plating efficiency assay. ⚫, TK6 cells cultured in non-selective medium (NS) prior to irradiation; ♦, TK6 cells cultured in HAT medium prior to irradiation. Error bars represent the standard error of the mean of the average of 3-5 replicate plates in two separate experiments.

Figure IV.7  **No evidence of large scale mutations at the hprt locus in 7 out of 11 radiation induced mutants**

Eleven 6-TG\textsuperscript{R} Co\textsuperscript{60} γ-irradiation induced mutants were cloned and tested for the presence of hprt genomic fragment (exon 8 - 9) by PCR analysis. A 1633 bp product indicated the presence of the human specific fragment, as demonstrated in TK6 DNA (lane 12). Of the 11 mutant clones tested (lanes 1-11), 4 show no evidence of any bands (lanes 8 -11). No bands were apparent in lane 13 containing no DNA. Restriction digested pCB10 was used as a molecular weight marker, lanes M.

Figure IV.8  **Confirmation of hprt and ts banding patterns of the 3 questionable radiation induced mutant clones**

Three 6-TG\textsuperscript{R} Co\textsuperscript{60} γ-irradiation induced mutants tested in lanes 8, 9 and 10 in Figure IV.8 were re-tested for the presence of hprt (1633bp) and ts (1689 bp) genomic fragments by PCR analysis. PCR of both gene fragments was simultaneously performed in the three clones and tested in lanes 1, 3 and 7 (representing clones M50.1, M50.2 and M300.1 respectively). EcoRV digested fraction of each sample was examined in lanes 2, 4 and 8 (representing clones M50.1, M50.2 and M300.1 respectively). Lanes 5 and 6 represent complementary results from a PCR reaction using TK6 DNA as a template. Restriction digested pCB10 was used as a molecular weight marker, lane M.
distinct band was present at 8 kbp in the parental TK6 cell line (positive control) (Figure IV.9). Further characterization of the 3 remaining candidate clones (Table IV.3) demonstrated that they were similar to the parental TK6 with respect to their doubling times, and their sensitivity to radiation (Table IV.3 and Figure IV.10). Upon testing for spontaneous reversion to the HPRT\(^+\) phenotype, no HAT\(^R\) clones were detected. The only observed difference between the 3 mutant clones was the spontaneous 7 day mutation frequency at the \(tk\) locus (corrected for plating efficiency) following TFT challenge.

**Table IV.3  Characterization of 6-TG\(^R\) mutants.**

<table>
<thead>
<tr>
<th>Clone</th>
<th>Doubling Times (h)</th>
<th>Radiation Sensitivity (D(_{37}), in rads)</th>
<th>Spontaneous Reversion to HAT(^R)</th>
<th>Spontaneous TK Mutation Frequency</th>
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</thead>
<tbody>
<tr>
<td>TK6</td>
<td>13.8</td>
<td>96</td>
<td>----</td>
<td>2 x 10(^{-5})</td>
</tr>
<tr>
<td>M50.1</td>
<td>11.8</td>
<td>97</td>
<td>(\leq 1 / 4.4 \times 10^6)</td>
<td>1 x 10(^{-4})</td>
</tr>
<tr>
<td>M50.2</td>
<td>12.5</td>
<td>61</td>
<td>(\leq 1 / 6.1 \times 10^6)</td>
<td>2 x 10(^{-5})</td>
</tr>
<tr>
<td>M300.3</td>
<td>13.4</td>
<td>81</td>
<td>(\leq 1 / 1.4 \times 10^6)</td>
<td>2 x 10(^{-5})</td>
</tr>
</tbody>
</table>

**4.2.4 SUMMARY**

As discussed in section 4.2.1, a large scale deletion at the \(hprt\) gene locus was needed so that the chances of spontaneous reversion and biased integration of the \(hprt\) minigene would be minimized. Methylcellulose-RPMI 1640 proved to be useful for mutation experiments employing anchorage independent TK6 cells. Establishing this methodology for scoring mutants expedited the experiments discussed in the following sections.
Figure IV.9  Further evidence of large scale deletions at the endogenous hprt locus in the 3 radiation induced mutant clones

Three 6-TG<sup>R</sup> Co<sup>60</sup> γ-irradiation induced mutants were re-tested for the presence of hprt genomic fragment (exon 5 - 7) by long-PCR analysis. An 8kp product indicated the presence of the human specific fragment, as demonstrated in TK6 DNA (lane 6). None of the 3 mutant clones tested (lanes 3, 4, and 5), show evidence of hprt bands. No bands were apparent in lane 7 containing no DNA. Lane 2 represents a λ PCR product of 16kb (positive control). Restriction digested λ and pCB10 (lanes 1 and 8 respectively) were used as a molecular weight markers.

Figure IV.10  Survival after Co<sup>60</sup> γ-irradiation of TK6 and 3 6-TG<sup>R</sup> mutant clones

Radiation killing effects were monitored by establishing the surviving fraction of irradiated cells, using the plating efficiency assay. All cells were cultured in non-selective RPMI medium prior to and for up to 20 days in non-selective methylcellulose-RPMI medium after irradiation. Error bars represent the standard error of the mean of the average of 5-7 replicate plates from a single experiments.
Cytogenetic analysis of TK6 metaphase spreads demonstrated a stable cell line with 2N = 46, with trisomy 13 and possibly some translocations, but only one X-chromosome (shown earlier by Yandell and Little, 1986). The doubling time of approximately 14 hours for the TK6 cell line was similar to literature reports of 14-18 hours (Liber et al., 1989; Amundson et al., 1993; Honma and Little, 1995).

With the confirmation that TK6 cell line had a stable karyotype, it was expected that the spontaneous mutation frequency at the hprt gene would be similar to that previously reported in this cell line (approximately 1-3 x 10⁻⁶) (Tachibana et al., 1990; Applegate et al., 1992; Oller and Thilly, 1992; Giver et al., 1993). Bleomycin treatment was expected to result in an increased number of recovered mutants (Povirk et al., 1994), observed in Table IV.1. The observation that spontaneous mutations in control (untreated) cells had a high mutation frequency (4 x 10⁻⁵) was unexpected. It was found that pre-treatment with impotent aminopterin was not effective in this experiment in eliminating pre-existing mutants from the test population. Increased background of mutants in this experiment was likely due to accumulation of spontaneous, small scale mutations.

From preliminary assessment of 5-azacytidine toxicity (Figure IV. 3) it was concluded that a 63 h treatment at 1 μM could allow testing of whether HAT₉ revertants could arise from the 6-TG₉ mutants. Eleven 6-TG₉ clones recovered after bleomycin treatment were likely not the result of methylation events since there was no reactivation by exposure to 5-azacytidine. The single clone that appeared to demonstrate phenotype reversion to HAT₉ (with or without 5-azacytidine treatment) was due to acquisition of
aminopterin resistance, as demonstrated by its clonability in the presence of 6-TG or the normally genotoxic aminopterin. Aminopterin resistance is a frequently observed drug resistant mutation occurring possibly through a mechanism involving *dhfr* gene amplification (Stark and Wahl, 1984; Gordon and Isola, 1993; Lücke-Huhle, 1994; Tlsty et al., 1995). A decrease in the expression of folate receptor or the carrier molecule for the reduced folate results in lowered intracellular levels of reduced folate and impaired aminopterin uptake, conditions which could also confer cellular drug resistance (Moscow et al., 1995). Despite the low plating efficiency of 5-azacytidine treated cells, it was concluded that the other 10 mutant clones were not the results of an epigenetic mutational event involving reversible *hprt* gene inactivation, as this phenotypic reversion has been described to occur at a frequency greater than $1 \times 10^{-3}$ (Holliday, 1991). The unexpectedly low plating efficiency may be due to an increased sensitivity of *hprt* mutants to 5-azacytidine, further discussed in section 4.5.4.

That methylation was likely not responsible for the loss of function of the *hprt* gene in the selected clones suggested that the bleomycin induced mutants were not epigenetic mutations. Further characterization of the selected mutants led to the observation that they all contained *hprt* specific genomic sequence. To ensure that the *hprt* sequences are not due to contaminating wild type TK6 cells, 14 clones were re-cloned and re-tested by PCR for the presence of the endogenous *hprt* gene. None of the 14 bleomycin-induced mutants appeared to be the result of a large scale mutational event.

Ionizing radiation was next tested as clastogenic mutagen to recover *hprt* deletion mutants. In this experiment, the observed spontaneous mutation frequency for TK6 cells
was 2.4 \times 10^{-6} and an inverse dose-dependent increase in induced mutation frequencies was observed for irradiated cells, as expected (discussed in section 4.4.4). Lack of PCR products for two different regions of the endogenous \textit{hprt} gene resulted in 3 potential candidates for further characterization and possible transfection. Further support for the suggestion that the \textit{hprt} gene was at least partially lost in the three mutants comes from the evidence that no spontaneous revertants from 6-TG\textsuperscript{R} to HAT\textsuperscript{R} were detected in any of the clones (more than $10^6$ viable cells tested for each clone).

The three clones were compared for their doubling times and radiation sensitivity. The assessment was made that the one with growth characteristics most similar to the parental TK6 had acquired the least amount of damage. No differences were observed in doubling times, spontaneous reversion frequencies or radiation sensitivity between the 3 different mutant clones. The final choice for which clone would be most suitable to be transfected with the \textit{hprt} minigene was based on the observed differences amongst clones in their spontaneous mutation frequency at the \textit{tk} locus. One of the mutant clones (M50.1) demonstrated a 5-fold increase in the number of spontaneously arising \textit{tk} mutants, suggesting possible mutator phenotype which would be inappropriate for future studies. Of the remaining clones, clone M50.2, a mutant induced by 50 rads, was chosen over clone M300.3, a mutant induced by 300 rads, on the assumption that this clone would have less overall DNA damage. Thus a clone demonstrating a stable 6-TG\textsuperscript{R} phenotype, possibly due to a large scale deletion, but retaining other desirable characteristics of the parental TK6 cell line, was chosen as the most suitable candidate for the transfection experiment described in section 4.3.
4.3 CREATION OF TRANSGENIC TK6 CELLS LACKING ENDOGENOUS \textit{hprt} BUT EXPRESSING TRANSFECTED HPRT

4.3.1 OBJECTIVES

The experimental strategy in developing a model system to monitor genomic instability was to transflect a suitable cell line with a marker gene (whose structure closely resembles that of the endogenous gene) which would integrate randomly in the genome. As described in section 4.2, a cell line was developed that displayed two important characteristics: i) no spontaneous reversion to the parental wild type phenotype; ii) no detectable PCR amplified products from two different regions of the endogenous gene. This suggested that most, if not all of the endogenous \textit{hprt} gene had been lost.

The minigene was transfected into the 6-TG$^R$ mutant (M50.2) by electroporation, a transfection method most likely to produce single-site, single-gene integrations. The human \textit{hprt} minigene marker was particularly suited for transfection into a human cell line: i) The minigene and the promoter comprised only human sequences. This ensures proper transcription of the integrated minigene since foreign sequences in the promoter region of minigenes have been associated with elevated levels of transgene mutations (Lichtenauer-Kaligis et al., 1993). ii) Just like the endogenous \textit{hprt} gene, the minigene could easily be selected for and against, thus allowing selection of forward and backward mutations. iii) Endogenous \textit{hprt} gene can easily be mutated to produce viable mutants,
which upon transfection would only express the functional \textit{hprt} minigene.

This section describes the derivation of cells expressing an \textit{hprt} gene following human minigene transfection by electroporation. The evidence that the minigene construct can be transcribed into a functional gene product comes from previous work with the mouse myeloma cell line SP2/0 (section 3.3.3.1), and was tested in the TK6 cell line by first looking for transient gene function upon transfection. A number of randomly selected permanent transfecants were characterized for appropriate transgene expression and transgene incorporation by Southern analysis.

4.3.2 METHODOLOGY

4.3.2.1 \textit{Purification of the hprt minigene}. The human \textit{hprt} minigene was introduced into the previously selected TK6 mutant clone M50.2 (section 4.2) by electroporation. The minigene fragment was excised from plasmid pHPT37-D2 using \textit{EcoRI} (Figure II.1), separated on an agarose gel by electrophoresis, recovered from the agarose and filter sterilized prior to use in electroporation for the construction of permanent transfecants. To minimize the cross contamination of the \textit{hprt} minigene (3.0 kb) with the pUC 8 vector fragment (2.7 kb), purification by gel electrophoresis was repeated. See sections 2.3 and 2.5 for more details.

4.3.2.2 Development of Electroporation conditions. To determine transfection efficiency and if the transgene was functional in the TK6 cell, the entire pHPT37-D2 plasmid was electroporated into 6-TG\textsuperscript{R} mutants (clone M50.2), and then, after 48 h of culturing in non-selective medium, cells expressing a functional product were selected in
HAT medium. Cell viability was assessed immediately following electroporation. Following 3 days in HAT medium, transient expression of the hprt minigene was determined by counting triplicate plates and back extrapolating to approximate survival upon challenge. To select permanent transfectants the hprt minigene (see section 4.3.2.1 and Appendix I) was electroporated into 6-TG\textsuperscript{R} mutants (clone M50.2). Estimates of the permanent transfection efficiency were determined after 14 days in HAT-selective methylcellulose-RPMI 1640 medium by scoring for visible colonies.

**4.3.2.3 Cloning of hprt transfectants.** In selecting permanent hprt transfectants, the electroporated cells were immediately subcultured in 96 well plates to eliminate sibling selection. After 5 a day expression period, the individual wells were challenged with HAT for up to 21 days. All wells with growth were re-cloned to minimize the possibility of selecting a mixed population. A total of 9 randomly selected and re-cloned permanent transfectants were further characterized by confirming their maintenance of HAT resistance by culturing them in HAT for one week and then monitoring for viability.

**4.3.2.4 Characterization of transfectants - Southern analysis.** Six different probes were tested for Southern analysis (see Figure II.2). Two different genomic digests of 9 transfected clones were tested for the presence of the transgene by Southern analysis. Each clone was digested with HindIII, an internal hprt minigene cutter (for expected Southern banding patterns see Figure IV.14a), or with EcoRV and SacI, which do not cut within the hprt minigene; therefore, the size of each band would be \( \geq 3.0 \text{ kb} \) and dependent upon closest external cut sites. DNA from 4 of the 9 clones was extracted at two different times, 5 months apart, and Southern analysis was repeated. Prior to each
extraction the cultures were selected in HAT to ensure the presence of a functional hprt
gene.

4.3.3 RESULTS

4.3.3.1 Development of Electroporation Conditions. pHPT37-D2 plasmid was
electroporated into TK6 mutant clone M50.2 to establish electroporation conditions and
ensure that the human hprt minigene can be expressed in this cell line. Cell viability in
this transfection experiment was estimated at approximately 50%. Recovery of transient
HAT\textsuperscript{R} transfectants, following electroporation with the complete pHPT37-D2 plasmid,
demonstrated a 28-fold increase over the expected number of surviving cells. This
increase was calculated from the number of control cells (electroporated in presence of no
DNA) surviving the HAT challenge after 3 days in selective medium. At 48 h post
exposure, it was estimated that the efficiency of transient transfection was between 2 and
3 %, assuming a 14 h doubling time for the electroporated TK6 cells and back-
extrapolating from the number of surviving cells on day 3 of HAT treatment to the
expected number of survivors on day 1 of the HAT challenge. A more permanent
transfection was estimated to be approximately 0.1% by long term culturing of
electroporated cells in HAT-selective methylcellulose-RPMI 1640 medium and scoring
for the number of long term surviving colonies.

4.3.3.2 Cloning of the HAT\textsuperscript{R} transfectants. A 3.0 kb EcoRI hprt minigene
fragment (Figure II.1) purified by two rounds of agarose gel electrophoresis (section
4.3.2.1) was introduced into 6-TG\textsuperscript{R} mutant clone M50.2 by electroporation. 10\textsuperscript{6} cells

-119-
were subjected to electroporation and viability was 26%. Culturing the surviving cells in 96 well plates ensured selection of independent transfectants; a total of 110 non-sibling clones was isolated. The success of permanent transfection in this experiment was estimated to be about 0.08%, based on 26% electroporation survival and a 50% plating efficiency. However, the true value was probably higher since it was possible that two independent transfectants may have survived the HAT challenge in a same well. For this reason, each randomly selected well demonstrating survival was re-cloned and subsequently retested for HAT resistance.

4.3.3.3 Choice of optimal sequences for probing of Southern blots. The first step in characterizing the 9 selected transfectants was to ensure that the phenotype switching of a TK6 cell from 6-TG$^R$ / HAT$^S$ to HAT$^R$ / 6-TG$^S$ was a consequence of transgene incorporation. Various attempts were made to perform Southern analysis using different probes. Sequencing of the pBluescript insert identified greater than 99% identity between the 335 bp hprt minigene XhoI / HindIII fragment and the reported hprt c-DNA (Edwards et al., 1990). Probes retaining the 5' GC rich region, or the remaining fragment of the intron 8 of the minigene bound non-specifically to genomic DNA, giving rise to background problems. The best probe (#6) that was capable of strong specific binding to the transfected minigene was specifically primed off exon 7 within the excised 335 bp hprt exon 3 - 7 fragment resulting in a probe of 330 bp.

4.3.3.4 Southern analysis. The 330 bp probe was hybridized to HindIII DNA digests of the 9 transfectants and the 2 parental cell lines (TK6 and M50.2). All 9 HAT$^R$ clones showed a transgene signal (Figure IV.11). This figure also demonstrates that a
Southern analysis of *HindIII* digested DNA from TK6, 6-TG<sup>r</sup> mutant (M50.2) and 9 HAT<sup>r</sup> transfected clones

Genomic DNA isolated from the parental TK6 cell line, the derived 6-TG<sup>r</sup> mutant (M50.2), 9 HAT<sup>r</sup> transfected clones (T164; T2411; T125; T259; T244; T236; T131; T1111; T238) and HL-60 (positive control human leukemic cells) was digested with *HindIII* and tested for presence of *hp* by Southern analysis. A 330bp minigene probe specifically annealed to the transfected *hp* fragments (exon 3-7) with some non-specific binding (possibly pseudogenes) in TK6, M50.2 and HL-60. Most transfectants also demonstrate a faint band at 3.3 kb, also present in TK6, M50.2, and HL-60. Strong bands, evident in all transfected clones, and unique for each transfectant, were supportive of the suggestion that the transfected fragments were integrated in different regions on the chromosomes. In a single integration transfectant digested with *HindIII*, a single band was expected to anneal to the probe resulting in an unpredictable size governed by an external *HindIII* cut site (clones T164; T2411 and T1111). Proposed patterns of *hp* minigene tandem integrations are presented in Figure IV.14b (clones T259; T244; T236; T131; and T238). Banding pattern of clone T125 and derived mutants (Figure IV.25a) combined with phenotypic instability of the transfected fragment, suggest an integration at two or possibly three different sites, with only one functional gene (further explanations in the text, page 134). The sizes of fragments were estimated by running a *HindIII* digest of pCB10 plasmid marker in a separate lane which was cut off prior to Southern hybridization.
weak band was detected in TK6 (wild type) DNA, but that the pattern of hybridization
was different than observed in the derived 6-TG\textsuperscript{R} mutant clone M50.2. Second Southern
analysis of DNA extracted from 4 transfected cell lines maintained in culture for an
additional period of 5 months demonstrated no change in banding patterns following
\textit{HindIII} digestion (data not shown).

When the same probe was used to hybridize to genomic DNA digested with
\textit{EcoRV} and \textit{SacI}, enzymes that do not cut within the \textit{hprt} minigene (Figure IV.12), 8 out
of the 9 transfectants demonstrated a presence of a single strong band while the ninth had
multiple bands (Figure IV.13).

4.3.4 SUMMARY

A highly purified fragment \textit{EcoRI} digested pHPT37-D2 was used to minimize any
contributions to genomic instability due to the presence of foreign sequences.
Electroporation with the complete plasmid pHPT37-D2 was first carried out to establish
conditions for electroporation. A low yield of transient transfectants (maximum of 3%),
and even a lower yield of permanent transfectants (0.1%) was found. However, this
experiment confirmed that the transfected plasmid encoding the \textit{hprt} minigene was
capable of producing a functional enzyme, as shown earlier (section 3.3.3.1).

Electroporation with the 3.0 kb minigene fragment or the entire circular plasmid
into a TK6 cell line lacking the endogenous \textit{hprt} gene resulted in a similar number of
permanent transfectants (0.08% and 0.1% respectively).

A GC rich region and repetitive sequences in intron 8 produced a high background
Figure IV.12  **Restriction digest map of the pHPT37-D2 to identify restriction enzymes that do not cut the hprt minigene**

Twelve different restriction digest enzymes were tested for their capability to internally cut the hprt minigene. Lanes 1 and 14 represent the pHPT37-D2 un-cut plasmid DNA. Lanes 2-13 represent different restriction digest enzymes (2, PvuI; 3, BclII; 4, ScaI; 5, StuI; 6, EcoRV; 7, HpaI; 8, SpeI; 9, BglII; 10, PstI; 11, SalI; 12, ScaI; 13, XbaI). pHPT37-D2 plasmid DNA remained intact in lanes 3-7 and 13. EcoRV and ScaI were selected for digesting genomic DNA in Figure IV.13.
Figure IV.13
Genomic DNA isolated from the parental TK6 cell line, the derived 6-TG<sup>R</sup> mutant (M50.2), 9 HAT<sup>R</sup> transfected clones (T164; T2411; T125; T259; T244; T236; T131; T1111; T238) and HL-60 was digested with EcoRV and SacI and tested for presence of hprt by Southern analysis. A 330 bp minigene probe annealed to the transfected hprt fragments (exon 3-7) with some binding (possibly pseudogenes) in TK6, M50.2 and HL-60. Strong bands, evident in all transfected clones, and unique for each transfectant, once again supported the suggestion that the transfected fragments were integrated in different regions on the chromosomes. The presence of single bands in clones T259, T244, T236, T131, and T238 with multiple copies of the transgene, suggests that all except clone T125, have integrated the hprt minigenes in tandem but clonally unique sites on the chromosomes. HL-60 once again demonstrated a different banding pattern, possibly suggesting different locations of endogenous or homologous sequences or different cut sites surrounding the regions that hybridize to the minigene fragment. All genomic fragment sizes were estimated from a separate lane that was run with HindIII digested pCB10 marker.
<table>
<thead>
<tr>
<th></th>
<th>TK6</th>
<th>M50.2</th>
<th>T164</th>
<th>T2411</th>
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<th>T259</th>
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</table>
in Southern analysis. Elimination of these produced a useful 330 bp probe encompassing exon 3-7. Four of the transfected clones were monitored for the *hprt* minigene banding pattern stability over time by Southern analysis. There was no apparent difference in the banding patterns of the 4 clones that expressed the gene after five months in culture. This apparent stability of the banding pattern suggests that the tandem repeats of the transgene were due to an early event, such as integration of tandem insertions or early amplification (as suggested by Windle et al., 1991), rather than an ongoing breakage and recombination event leading to gene amplification.

Figure IV.14a shows the fragments expected to be detected on a Southern blot corresponding to the *hprt* minigene tandem insertions. Proposed orientation of tandem integrations of the 5 independent transgenic clones, based on the observed fragment size estimates, are presented in Figure IV.14b. Testing of the different genomic digests (Figure IV.11) led to the conclusion that only 3 (T164, T2411 and T1111) out of the 9 transfectants examined harbour a single copy transgene. The hybridized fragments in these 3 clones were of different sizes, supporting the suggestion that the sites of integration may have been different in each of the three clones. The remaining 6 clones appear to have incorporated multiple copies of the transgene (Figures IV.11 and IV.14b). Five out of the 6 clones (T259, T244, T236, T131 and T238) demonstrated single-site tandem repeats, suggested by the presence of contiguous fragments when DNA was digested with external cutters (Figure IV.13). Clone T125 was possibly a clone with up to 3 different insertion sites, or alternatively, 2 sites of integration with one having a single copy and the second one having two genes in tandem. The presence of a banding
Figure IV.14a  Expected fragments detected by Southern analysis corresponding to different possible hprt minigene insertions

Tandem insertions of the hprt minigene are expected to result in a banding pattern representative of a head-to-tail, tail-to-tail, head-to-head or a combination of the three orientations. Based on the hprt minigene size (3.0 kb), the internal cutter used to digest the minigene (HindIII) and the 330 bp minigene probe (exon 3-7), three different configurations are expected:  i) A head-to-tail integration, probed with hprt minigene sequence upstream of the HindIII cut site, is expected to give a 3.0 kb and an indeterminate size bands. ii) A tail-to-tail tandem integration, probed with the same hprt minigene sequence, is expected to give 2 indeterminate size bands. iii) A head-to-head tandem integration, probed with the hprt minigene sequence, is expected to give a single band of 1.4 kb.

Figure IV.14b  Proposed arrangement of tandem insertions of the hprt minigene based upon observed Southern analysis banding patterns

Based upon the observed banding patterns from Figure IV.11, and the expectations from Figure IV.14a, proposed arrangements for multiple transgene integrations are presented. Clone T259 is expected to house a tail-to-tail tandem insertion. Clone T244 appears to have 4 genes in tandem, where 2 identical head-to-tail arrangements are annealed tail-to-tail. The banding patterns observed for clones T236 and T131 can be explained by a tail-to-tail and a head-to-tail arrangement. Clone T238 appears to have two genes in tandem with a tail-to-tail orientation. For all clones the observed calculated fragment length are shown.
Legend for the inserted hpRT minigene

Region detected by 330 bp probe

![Diagram of the inserted minigene and the expected fragment detectable by Southern analysis.]

<table>
<thead>
<tr>
<th>Figure IV.14a</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) 5' - 3' - 5' - 3'</td>
</tr>
<tr>
<td>2) 5' - 3' - 3' - 5'</td>
</tr>
<tr>
<td>3) 3' - 5' - 5' - 3'</td>
</tr>
<tr>
<td>3 kb</td>
</tr>
<tr>
<td>1.4 kb</td>
</tr>
</tbody>
</table>

Figure IV.14b

Clone T259

5' - 3' - 5' - 3' - 5' - 2.58 kb
7.74 kb

Clone T244

5' - 3' - 5' - 3' - 5' - 3.31 kb
1.51 kb
2.89 kb

Clone T236

5' - 3' - 3' - 5' - 3.29 kb
1.85 kb
2.87 kb

Clone T131

5' - 3' - 3' - 5' - 6.13 kb
5.29 kb
2.90 kb

Clone T238

5' - 3' - 3' - 5' - 2.81 kb
4.34 kb

-126-
pattern in the wild type TK6 and its derived mutant, M50.2 (Figures IV.11 and IV.13), is likely to be a consequence of probes annealing to one of the 4 pseudogenes known to be present in the human genome, since most of the endogenous *hprt* gene was believed to be deleted. However, it is possible that the endogenous exon 3, the largest of the *hprt* exons (184 bp), was still present, and capable of annealing to the probe.

Examination of the 5 transfectants with tandem insertions showed no evidence of functional 5' - 5' (head-to-head) integrations. Of the 9 *hprt/hprt* boundary regions examined (Figure IV.14b), the integrated sequences were arranged in a 3' - 3' (tail-to-tail) in five and 5' - 3' (head-to-tail) configuration in four cases. If the assumption is made that the reason for tandem insertions is the random annealing of fragments prior to integration into the chromosomes, than the expectation would be that each configuration would have an equal chance of occurring. This randomness would be disrupted if the tandemness was a consequence of post-insertional amplification of single transgene insertions, or if certain configurations were not functional. Table IV.4 presents the expected (based on fragments annealing prior to integrations) and the observed configurations of the tandem inserts.

<table>
<thead>
<tr>
<th>Configuration of tandem insertions</th>
<th>Expected occurrence</th>
<th>Observed occurrence</th>
</tr>
</thead>
<tbody>
<tr>
<td>3' - 5' and 5' - 3'</td>
<td>50%</td>
<td>4/9 = 44%</td>
</tr>
<tr>
<td>3' - 3'</td>
<td>25%</td>
<td>5/9 = 56%</td>
</tr>
<tr>
<td>5' - 5'</td>
<td>25%</td>
<td>0/9 = &lt; 0.1%</td>
</tr>
</tbody>
</table>
With these small numbers of boundary regions examined and no evidence to show that the head-to-head orientation is functional, it is impossible to determine whether a bias for post-insertional amplification of transgenes exists or whether genomic fragments anneal to each other prior to integration into the chromosomal DNA giving rise to transfectants with a 67% probability of tandem inserts (6 of the 9 clones tested).

However, it is clear that integrants harbouring a single-site, single-gene insertion are in the minority.
4.4 SPONTANEOUS AND INDUCED MUTATION FREQUENCIES OF THE TRANSFECTANTS AND THE PARENTAL TK6 CELL LINE

4.4.1 OBJECTIVES

Having selected independent transfecteds (section 4.3), the next objective was to compare the stability of the hprt transgene in the 9 different clones by monitoring the accumulation of spontaneous or induced events that resulted in phenotype switching. The assumptions are that the site of transgene integration is random and that the marker of mutational events is unaltered at the time of integration.

In calculating the spontaneous mutation frequencies, 9 transfected clones were monitored for loss of transgene function over a period of up to 28 days. To eliminate the possibility that differences in mutation frequencies were due to the differences in the cell doubling times, results are expressed as mutation rates, that is mutation frequency divided by the number of cell divisions.

High spontaneous mutation rate was found in most clones; this could have been due to the presence of a general mutator phenotype. To examine this possibility further, mutation at a second independent site was monitored. The spontaneous mutation rate at the endogenous heterozygous tk gene marker of the TK6 parent was compared with the mutation rate found in the 9 transfected cell lines. In determining induced mutation frequencies, irradiation using Co^{60} γ-rays was employed as a clastogenic mutagen. An
attempt was made to increase the number of surviving mutants following clastogenic
damage by abrogating G₂ cell arrest with caffeine treatment (Zhen and Vaughan, 1995;
Zhen et al., 1995). Increasing the mutant recovery might improve the model for studying
clastogen induced mutational events.

4.4.2 METHODOLOGY

4.4.2.1 Spontaneous mutation frequencies. In establishing spontaneous
mutation frequencies in the 9 transfected clones and the 2 predecessors (TK6 and M50.2),
each cloned population was subjected to appropriate selective medium to reduce the
background mutation frequency. To do this, TK6 and the 9 transfected cell lines were
selected in CHAT for 7 days, maintained in CHT for 2 days, and then subcultured to non-
selective medium (day 0) for up to 35 days to isolate mutants. Clone M50.2 was selected
in 6-TG for 7 days, subcultured to non-selective medium for 2 days, and maintained, in
non-selective medium, for the remainder of the test period. All cultures were maintained
at sub-saturation levels. Starting on day 1, each population was sampled at regular
intervals to determine plating efficiencies in non-selective medium, and mutation
frequencies in selective-methylcellulose-RPMI 1640 medium. Functional HPRT and TK
were detected with HAT and CHAT, respectively, while mutant hprt and tk were detected
with 6-TG and TFT, respectively. Viable colonies were scored on day 14 or 21 post-
challenge. Mutation frequencies at each time point were expressed as the number of
mutant colonies per number of viable cells, calculated from plating efficiencies for each
sample. Mutation frequencies were then converted to mutation rates by applying
doubling time information that was determined for each clone.

4.4.2.2 Radiation induced mutation frequencies. In determining the radiation-induced mutation frequencies, all samples were pre-treated in a similar manner, as described above. On day 1, fractions of each culture were irradiated by different doses of Co\textsuperscript{60} \(\gamma\)-rays, as described in section 2.4.2. Immediately following irradiation, survival was estimated by establishing plating efficiencies. The remainder of the exposed and non-exposed cultures were maintained under non-selective conditions for 7 days to allow phenotypic expression. On day 8, a fraction of each culture was assessed for mutation frequency and plating efficiency, as described above. Induced mutation frequencies were calculated after correcting for spontaneous mutation frequencies.

4.4.2.3 Radiation induced mutation frequencies in the presence of caffeine.

Parental TK6 cells, selected in CHAT, were pre-treated (\pm caffeine) for 1 h before irradiation and approximately 1 h after irradiation (described in section 2.4.5). A dose response curve and mutation frequencies for \(\pm\) caffeine treated samples were determined.

4.4.3 RESULTS

4.4.3.1 Doubling time of TK6 and derived clones. In order to present all mutation frequencies as mutation rates, doubling times were established from linear regression analysis of the growth curves for all 9 transfected clones plus TK6 and M50.2 (Figure IV.15a and b). Clone T244 demonstrated the fastest generation time (12.7 h), while clone T1111 was the slowest (14.6 h). Table IV.5 presents individual doubling times used in converting mutation frequencies to mutation rates.
Figure IV.15  Growth curves of TK6, 6-TG<sup>R</sup> mutant (M50.2) and 9 HAT<sup>R</sup> transfected clones

Growth curves of different clones and transfectants was determined by performing haemocytometer counts, as described in section 2.2.3. The number of viable cells was scored and plotted as a function of time. Each data point is a mean of at least two counts from three representative culture plates (±S.E.M.). Figure IV.15a presents data for clones; TK6, M50.2, T125, T131, T1111 and T164. Figure IV.15b presents data for clones; TK6, T244, T259, T236, T238 and T2411. Statistical analysis of the different growth curves was performed by the SAS (Statistical Analysis Social Sciences) program. Analysis of 198 unpaired independent data points using the F test (independent estimates of variance) gave a difference between the slopes of the growth curves that was considered to be a significant (P = 0.00362).
Table IV.5  Estimated doubling times of TK6 and derived clones

<table>
<thead>
<tr>
<th>TK6</th>
<th>M50.2</th>
<th>T164</th>
<th>T2411</th>
<th>T125</th>
<th>T259</th>
<th>T244</th>
<th>T236</th>
<th>T131</th>
<th>T1111</th>
<th>T238</th>
</tr>
</thead>
<tbody>
<tr>
<td>13.8 h</td>
<td>14.0 h</td>
<td>13.8 h</td>
<td>14.3 h</td>
<td>13.8 h</td>
<td>13.4 h</td>
<td>12.7 h</td>
<td>14.5 h</td>
<td>14.2 h</td>
<td>14.6 h</td>
<td>14.2 h</td>
</tr>
</tbody>
</table>

4.4.3.2 Spontaneous mutation frequencies. Spontaneous *hprt* and *tk* mutation frequencies were determined for the parental TK6 and 9 transfected clones. The mutant clone M50.2 was monitored for reversion to HAT<sup>R</sup> phenotype and ≤ 1 colony per 3 x 10<sup>6</sup> cells was seen (consistent with previous results, section 4.2.3.7). Spontaneous mutation frequencies at the endogenous *hprt* (TK6) and the transfected *hprt* gene (9 transfectants) was examined (Figure IV.16) (note change of scale in the second part of the figure). A wide range of the number of mutants recovered over a period of up to 29 days was seen. In contrast, the same 10 clones appeared similar in the spontaneous mutation frequencies at the *tk* locus (Figure IV.17) (note change of scale in the second part of the figure). When all data were converted to spontaneous mutation rates (correcting for differences in doubling time) an increase of up to 2000-fold was observed at the *hprt* gene in some of the transfectants compared to the parental TK6 (Figure IV.18). Spontaneous mutation rates demonstrated a range of a 1000-fold within the different transgenic cell lines. Even the most stable transgenic cell lines demonstrated a 2-fold increase in the mutation rate at the *hprt* transgene when compared to the endogenous *hprt* gene in the TK6 cell line. In contrast, an increase of only 7.5-fold was observed at the *tk* gene in some of the transfectants when compared to the parental TK6 line, with a range of 30-fold between all clones tested (Figure IV.19). Table IV.6 presents the summarized data from figures IV.18 and IV.19.
Figure IV.16  Spontaneous mutation frequencies at the endogenous or transfected *hprt* locus

Spontaneous mutation frequencies of TK6 and 9 transfectant clones were determined by periodically challenging a TK\(^{-}/\)HPRT\(^{-}\) population of cells cultured under non-selective conditions for up to 29 days and monitoring for appearance of 6-TG\(^{R}\) mutants. At each time point a fraction of the population was removed for mutation frequency testing in selective methylcellulose-RPMI medium and for plating efficiency determination in non-selective medium. Viable colonies were scored on days 21 and 14, respectively for mutation frequency and plating efficiency tests. Each data point represents the mean of colony counts from 3 separate cultures (±S.E.M.) corrected for viability. Top figure presents data for clones TK6, T164, T2411, T125, T259 and T244. Bottom figure presents data for clones T236, T131, T1111 and T238.
Figure IV.17  Spontaneous mutation frequencies at the endogenous tk locus

Spontaneous mutation frequencies of TK6 and 9 transfectant clones were determined by periodically challenging a TK+/HPRT- population of cells cultured under non-selective conditions for up to 29 days and monitoring for appearance of TK- mutants with TFT. At each time point a fraction of the population was removed for mutation frequency testing in selective methylcellulose-RPMI medium and for plating efficiency determination in non-selective medium. Viable colonies were scored on days 21 and 14, respectively for mutation frequency and plating efficiency tests. Each data point represents the mean of colony counts from 3 separate cultures (±S.E.M.) corrected for viability. Top figure presents data for clones TK6, T164, T2411, T125, T259 and T244. Bottom figure presents data for clones T236, T131, T1111 and T238.
Figure IV.18  Spontaneous mutation rates at the endogenous or transfected \textit{hprt} locus

Linear regression analysis of spontaneous mutation frequencies observed in Figure IV.16 were converted to mutation rates by correcting the extrapolated rates for generation doubling times.

Figure IV.19  Spontaneous mutation rates at the endogenous \textit{tk} locus

Linear regression analysis of spontaneous mutation frequencies observed in Figure IV.17 were converted to mutation rates by correcting the extrapolated rates for generation doubling times.
Table IV.6  Summary of spontaneous mutation rates at the hprt and the tk locus*

<table>
<thead>
<tr>
<th></th>
<th>TK6</th>
<th>T164</th>
<th>T2411</th>
<th>T125</th>
<th>T259</th>
<th>T244</th>
<th>T236</th>
<th>T131</th>
<th>T1111</th>
<th>T238</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPRT</td>
<td>2</td>
<td>4.1</td>
<td>4.2</td>
<td>17.6</td>
<td>42.4</td>
<td>59.1</td>
<td>1103</td>
<td>1239</td>
<td>2979</td>
<td>3801</td>
</tr>
<tr>
<td>TK</td>
<td>8</td>
<td>2</td>
<td>5</td>
<td>3</td>
<td>22</td>
<td>53</td>
<td>2</td>
<td>8</td>
<td>60</td>
<td>37</td>
</tr>
</tbody>
</table>

*All values represent # of mutants/10^7 cells/generation

4.4.3.3 **Radiation induced mutation frequencies.** Mutation frequencies were established for each clone, following Co<sup>60</sup> γ-irradiation, to determine their relative sensitivities for detecting clastogenic events. In agreement with previous reports (Konig and Kiefer, 1988; Amundson and Chen, 1996), at higher radiation doses (at approximately 120 rads) a decrease in both hprt and tk mutants was observed (Figure IV.20). The hprt mutant clone M50.2, was monitored for reversion to the HAT<sup>R</sup> phenotype following Co<sup>60</sup> γ-irradiation and no viable colonies were detected among 3 x 10<sup>6</sup> viable cells tested.

4.4.3.4 **Radiation induced mutation frequencies in the presence of caffeine.** Caffeine did not produce a dose-dependent increase in cell survival following 130 rads of Co<sup>60</sup> γ-irradiation (Figure IV.21). An examination of the hprt mutation frequencies suggested that, when compared to irradiated cells alone, 2 mM caffeine plus irradiation resulted in a 6-fold increase in the number of recovered mutants (Figure IV.22). Moreover, exposure to caffeine alone did not produce a detectable increase in the number of mutants (Figure IV.22).

4.4.3.5 **Ratio of spontaneous to induced mutation frequencies.** In considering spontaneous and induced mutation frequencies, most cells exhibit a dose related increase
Figure IV.20  Radiation induced mutation frequencies at the endogenous or transfected *hp*rt locus and the endogenous *tk* locus

Induced mutation frequencies of TK6 and 9 transfectant clones were determined by irradiating TK\(^+\)/HPRT\(^-\) selected population of cells and monitoring for appearance of HPRT\(^-\) mutants in 6-TG (upper graphs) or TK\(^-\) mutants in TFT (lower graphs) after a 7 day expression period in non-selective medium. A fraction of each population was tested for plating efficiency in non-selective medium. Viable colonies were scored on days 21 and 14, respectively for mutation frequency and plating efficiency tests. Each data point represents the mean of colony counts from three separate cultures (±S.E.M.) corrected for viability and spontaneous mutation frequencies.
HPRT MUTANTS / 10^6 VIABLE CELLS

DOSE (RADS)

TK MUTANTS / 10^6 VIABLE CELLS

DOSE (RADS)
Figure IV.21  Survival of TK6 lymphoblastoid cells in the presence of different concentrations of caffeine after Co\textsuperscript{60} \(\gamma\)-irradiation

TK6 cells, treated with different concentrations of caffeine (one hour before treatment, for a total of 2 hours) were monitored for survival by establishing plating efficiencies of treated cells immediately after 130 rads of Co\textsuperscript{60} \(\gamma\)-irradiation. Data points at each concentration represent the fraction of mean number of clonable cells to total number of cells plated from 9 different culture plates scored after a 14 to 21 day incubation period (±S.E.M.). The variation among different treatments is not significantly greater than expected by chance (\(P=0.1177\) calculated by nonparametric ANOVA test).

Figure IV.22  Mutation frequency at the hprt endogenous locus in the TK6 lymphoblastoid cell line in the presence of different concentrations of caffeine after Co\textsuperscript{60} \(\gamma\)-irradiation

TK6 cells, treated with different concentrations of caffeine (one hour before treatment, for a total of 2 hours) were monitored for mutation frequency and plating efficiency of treated cells 7 days after 130 rads of Co\textsuperscript{60} \(\gamma\)-irradiation. Parallel data points for non-irradiated and irradiated TK6 cells at each concentration of caffeine are represent as the mean viable mutant fraction of cells plated from 9 different culture plates scored after a 14 to 21 day incubation period (±S.E.M.). The variation among different concentrations of caffeine is not significantly greater than expected by chance (\(p=0.1439\) calculated by nonparametric repeated measures test). The variation within the same concentrations (± irradiation) is also not significant (one tailed P value is 0.1386 calculated by a nonparametric test)
in mutation frequencies with radiation. When this ratio was examined at day 7 after irradiation treatment in the TK6 cell line and the derived transfectants (Figure IV.23), a small increase was observed in induced mutational events at the tk locus. However, when the same ratio was examined at the hprt gene locus (Figure IV.24), the contribution from induced mutational events appeared insignificant for some of the clones that demonstrated high spontaneous mutation. Clone T1111 and T238 demonstrated high spontaneous and induced mutation frequencies that were above saturation limits for clonal detection and thus must be considered to underestimate the real values.

4.4.4 SUMMARY

A number of different clones were monitored for spontaneous mutation frequencies over a period of up to 29 days. Large differences in mutation frequencies between the clones were observed at the hprt gene, compared to only small differences at the tk locus. This suggests that the site/number of transgene integration may affect the stability of the transgene. The capability of monitoring the mutation frequency at the tk gene locus provides evidence that the elevated levels of hprt mutations were not a consequence of generalized mutator phenotype causing genomic instability. The 7 day mutation frequencies at the tk gene locus, observed in these experiments, closely correlate with previous reports of 3 to 10 x 10⁴ (Thilly et al., 1980; Chaudhry et al., 1996).

All mutation frequency data were converted to mutation rates. The clones that demonstrated the highest spontaneous mutation rate at the hprt gene locus did not show a similar increase in mutation rate at the tk gene locus (Table IV.6). Normalizing for the
Figure IV.23  A ratio of background spontaneous to Co$^{60}$ γ-irradiation induced mutation frequencies at the $tk$ endogenous locus in the TK6 lymphoblastoid cell line and 9 transfectants

Day 7 spontaneous mutation frequencies of TK6 and 9 transfectants were compared to day 7 Co$^{60}$ γ-irradiation induced mutation frequencies for the same clones. The data for this figure were extrapolated from Figures IV.17 and IV.20 (Lower graphs).

Figure IV.24  A ratio of background spontaneous to Co$^{60}$ γ-irradiation induced mutation frequencies at the endogenous or transfected hprt locus in the TK6 and 9 transfectants

Day 7 spontaneous mutation frequencies of TK6 and 9 transfectants were compared to day 7 Co$^{60}$ γ-irradiation induced mutation frequencies for the same clones. The data for this figure were extrapolated from Figures IV.16 and IV.20 (Upper graphs).
mutation rates at the tk locus, it was concluded that mutation rates were variable among hprt transgenic clones.

The two transfected clones demonstrating the most stable hprt transgenes (T164 and T2411) were found to have a 2-fold increase in mutation rates (approximately 4/10^7 cells/generation) when compared to the stability of the endogenous hprt in the parental TK6 line (2/10^7 cells/generation). These same 2 clones, demonstrating the most stable transgene phenotype, were also the 2 clones where a single copy of the transgene was integrated at a single site in the genome. However, taking into consideration the 19-fold difference in the gene target size between the endogenous and the transfected marker (57 kb versus 3.0 kb respectively), the expectation was that the transgene would accumulate spontaneous chromosomal mutations at a somewhat slower rate.

Table IV.6 demonstrates that the two clones (T164 and T2411) were at least 4 times more stable in the expression of their transgene than the next most stable transfectant, clone T125 (with 3 inserts), 10 times more stable than clone T259 (which demonstrated a tail-to-tail tandem insertion) and 300 times more stable than clone T131 (a possible orientation of head-to-tail-to-tail of 3 tandem transgenes). Clone T125, demonstrating 3 specific bands in Figure IV.11a and at least 3 in Figure IV.13a, suggested a presence of more than one copy of the transgene at possibly 2 or 3 different sites. The relative ease of detecting spontaneous mutants of this clone, 5 fold higher than that observed for clones T164 and T2411, suggested that only one of the transgenes was functional. Support for this suggestion comes from the observations to be reported in the next section (4.5.3.2, and Figure IV.25), where all mutants from clone T125 are due to the
loss of a single band. The two remaining bands appear unchanged in the 6-TG\textsuperscript{R} phenotype mutants, suggesting that they were initially non-functional. Clones T1111 and T238, which demonstrated the highest spontaneous mutation rates, were excluded from these comparative analysis as explained in section 4.5.4.

Attempts to attenuate G\textsubscript{2} checkpoint control by caffeine pre-treatment demonstrated no increase in survival after irradiation of TK\textsubscript{6} cells. However, 2 mM caffeine pre-treatment appeared to increase the number of hprt mutants surviving irradiation. This increase in the number of mutants was not attributed to caffeine acting as a mutagen since equivalent concentrations of caffeine without irradiation did not result in an increased number of recovered mutants.
4.5 CHARACTERIZATION OF SPONTANEOUS 6-TG<sup>R</sup> MUTANTS OF THE TRANSFECTANT SUBCLONES

4.5.1 OBJECTIVES

The finding that most transfected genomic sequences were less stable than the endogenous genomic sequences led to characterization of a number of mutant clones that lost the transgene function. Some had very high spontaneous mutation frequency. This could be attributed to inactivation of genomic sequences by hypermethylation or to recombinational loss of the transfected sequences due to the inherent instability at the site of integration. This chapter examines several clones for the cause of this transgene instability.

The possibility that newly integrated genomic sequences may undergo changes in methylation was tested by treating a number of mutants from each transgenic clone with 5-azacytidine to test for reactivation. The observation that single gene insertions are more stable (section 4.4.3.2) suggests that they might also be less susceptible than tandem insertions to inactivation by methylation. As discussed in section 1.5.1, tandem insertions are known to be highly unstable.

In this chapter, Southern analysis was performed on different revertant clones (recovered from transfectants, see Appendix I for nomenclature) to determine if large scale deletions were causing transgene inactivation. The 5 transfectants with tandem insertions (Table IV.6; clones T125, T259, T244, T236, and T131) demonstrated
spontaneous inactivation/loss of a transgene at a rate 4 to 300-fold higher than that observed in a single transfectant clones.

4.5.2 METHODOLOGY

4.5.2.1 Phenotype reversion by 5-azacytidine. An attempt was made to reactivate the spontaneously arising 6-TG<sup>R</sup> mutants by 63 h exposure to 5-azacytidine, as described in section 2.4.4 and 4.2.3.4. For each of the 9 transfected clones and the parental TK6 cell line, a number of mutants were pooled and treated with 5-azacytidine to determine if there were any phenotype revertants upon subsequent challenge with HAT. Simultaneously, a parallel experiment was set up to test for the possibility of spontaneous reversion to HAT<sup>R</sup> phenotype. All pooled cultures of 6-TG<sup>R</sup> mutants, ± 5-azacytidine at concentrations of 1 or 3 μM, were monitored for growth in the presence of non-selective medium and for reversion in HAT medium (see sections 2.2.2 and 2.4.4). Positive cultures were subsequently tested for resistance in 4 x 10<sup>-7</sup> M aminopterin (i.e., without hypoxanthine and thymidine).

4.5.2.2 Southern analysis of phenotype reversion mutants. Southern analysis was used to examine 5 to 6 different 6-TG<sup>R</sup> mutant clones arising from spontaneously occurring events in each of the 9 transfected clones cultured for 35 days under non-selective conditions. The protocol used to detect the transgenic sequences was described in sections 2.5.6 and 4.3.2.4. Briefly, DNA isolated from different mutants was digested with Hind III enzyme, fractionated on an agarose gel and hprt gene specific fragments were detected by hybridization with probe #6 (Figure II.2b).
4.5.3 RESULTS

4.5.3.1 Induction of phenotype reversion by 5-azacytidine. Two to 4 pooled samples of 6-TG\(^R\) mutants, consisting of different numbers of individual colonies presented in Table IV.7, were tested for reactivation of the transfected gene by the demethylating agent, 5-azacytidine.

Table IV.7 5-Azacytidine treated pools of 6-TG\(^R\) mutants tested for phenotype reversion.*

<table>
<thead>
<tr>
<th>Clone</th>
<th># of 6-TG(^R) (HPRT(^+)) Colonies Per Pool</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pool A</td>
</tr>
<tr>
<td>TK6</td>
<td>2</td>
</tr>
<tr>
<td>T164</td>
<td>2</td>
</tr>
<tr>
<td>T2411</td>
<td>3</td>
</tr>
<tr>
<td>T125</td>
<td>14</td>
</tr>
<tr>
<td>T259</td>
<td>3</td>
</tr>
<tr>
<td>T244</td>
<td>1</td>
</tr>
<tr>
<td>T236</td>
<td>3</td>
</tr>
<tr>
<td>T131</td>
<td>27</td>
</tr>
<tr>
<td>T1111</td>
<td>58</td>
</tr>
<tr>
<td>T238</td>
<td>45</td>
</tr>
</tbody>
</table>

* Pools A and B recovered after 3 \(\mu\)M and pools C and D after 1 \(\mu\)M treatments.

Plating efficiencies of the pooled samples exposed to 5-azacytidine were 5\% and 0.5\% for the 1 and 3 \(\mu\)M concentrations, respectively, compared to the 50\% observed in the non-treated controls. Challenging 3 \(\times\) 10\(^5\) cells from each 5-azacytidine treated cultures of pooled sample with HAT (selecting for reversion of \(hprt\) expression)
demonstrated that mutants from 2 of the 9 clones (T1111 and T238) were capable of 37% and 96% survival, respectively, in the selection medium. However, the 6-TG\textsuperscript{R} mutants from these 2 respective clones were also capable of 44% and 58% survival in HAT medium, without the pre-treatment in 5-azacytidine. Subsequent culturing of the same pooled populations also demonstrated 28% and 70% survival in aminopterin alone, indicating that aminopterin resistance rather than \textit{hprr} gene reactivation had occurred. Challenge of all other pooled mutants following 5-azacytidine treatment of 3.3 x 10\textsuperscript{4} viable cells from each population, resulted in the isolation of only 2 HAT surviving colonies from clone T2411 while no spontaneously arising HAT\textsuperscript{R} colonies were detected in a total of 5 x 10\textsuperscript{3} viable T2411 mutant cells tested. The 2 HAT\textsuperscript{R} 5-azacytidine induced colonies, derived from T2411, did not grow in aminopterin alone without the presence of hypoxanthine and thymidine.

\textbf{4.5.3.2 Southern analysis of reversion mutants.} Southern analysis was used to examine 41 spontaneously arising revertants from different transfectant clones (Figure 25a-c). All revertants demonstrated deletions at the \textit{hprr} transgene. Twenty two had lost all \textit{hprr} hybridizing bands and 18 showed partial deletions. None of the revertants demonstrated identical banding pattern to that observed in the parental cell line. One revertant clone (#4) isolated from clone T2411 (Figure 25a) did not demonstrate any non-specific bands upon hybridization, suggesting lack of DNA in that lane. Probability estimates, obtained by the use of a statistical simulation program, examined the likelihood that the analysed mutants are population siblings (Table IV.8). This statistical simulation program was designed by Dr. M. Goddard, Health Canada, Environmental Health
**Figure IV.25a  Southern analysis of HAT\textsuperscript{R} transfectants and 6-TG\textsuperscript{R} reversion mutants**

*HindIII* digested DNA from clones T164, T2411, T125 and T259 demonstrate the banding patterns for the HAT\textsuperscript{R} transfectant parent clones in the first lane and 6 spontaneous, randomly selected 6-TG\textsuperscript{R} mutants. All Southern blots were probed with the 330 bp exon 3 - 7 fragment of the hprt minigene (described in Figure II.2b, #6). **T164** derived mutants demonstrate no evidence of the parental transgene band at 4.44 kb, but a faint presence of non-specific bands is apparent. **T2411** shows 6 mutants with deletions. Lanes 1, 2, and 3 show approximately a 170 bp deletion and clones 5 and 6 have approximately a 280 bp deletion in the expected band of 5.29 kb. Lane 4 mutant has lost the parental band despite the presence of faint non-specific binding. **T125** demonstrates 5 of the 6 mutants with identical banding pattern to the parental clone (8.73 kb and 1.28 kb), except for missing a single band at 3.04 kb (lanes 1,2,4,5 and 6). Mutant in lane 4 has a single remaining band at 10.2 kb. 6 mutants from **T259** show total loss of both parental bands (7.74 kb and 2.58 kb).
Figure IV.25b  Southern analysis of HAT$^R$ transfectants and 6-TG$^R$ reversion mutants

Clones T244, T236, T131 and T1111 demonstrate the banding patterns for the HAT$^R$ transfectant parent clones and 5 to 6 spontaneous, randomly selected 6-TG$^R$ (HPRT$^-$) mutants. All DNA was digested with HindIII and probed with a 330 bp hprt minigene exon 3 - 7 fragment (Figure II.2b). T244 derived mutants demonstrate no evidence of the transgene (3.33 kb, 2.88 kb or 1.51 kb) but a faint presence of non-specific bands in 5 of the 6 mutants tested. Mutant in lane 2 appears to have a single remaining band of 1.65 kb. T236 shows 5 mutants with identical banding pattern, where 2 of the 3 parental bands have been lost (2.88 kb and 1.85 kb). T131 demonstrates 5 of the 6 mutants with complete loss of all bands (6.13 kb, 5.29 kb and 2.88 kb). Clone in lane 5 shows a single remaining band at 10.5 kb. T1111 appears to have lost the parental band (9.93 kb) in 2 out of the 5 mutant clones tested (lanes 3 and 5).
Figure IV.25c  Southern analysis of HAT$^R$ transfectants and 6-TG$^R$ reversion mutants

*Hind*III digested DNA was probed with a 330 bp exon 3 - 7 fragment of the *hprt* minigene (Figure II.2b). T238 demonstrates the banding patterns for the HAT$^R$ transfectant parent clones and 4 spontaneous, randomly selected 6-TG$^R$ mutants. All 4 demonstrate an identical banding pattern to that observed in the parental HAT$^R$ clone (4.34 kb and 2.88 kb bands).
Division, and is presented in Appendix III. Mutants recovered from clone T1111 and T238 were not included in this comparative analysis due to their inherent resistance to drug selection (aminopterin) which resulted in enhanced recovery of spontaneous 6-TG$^R$ mutants in subsequent populations.

<table>
<thead>
<tr>
<th>Clone</th>
<th>Mean of the Simulated Probabilities ± S.E.M (N=100)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TK6</td>
<td>0.158 ± 0.006</td>
</tr>
<tr>
<td>T164</td>
<td>0.094 ± 0.005</td>
</tr>
<tr>
<td>T2411</td>
<td>0.094 ± 0.004</td>
</tr>
<tr>
<td>T125</td>
<td>0.023 ± 0.0006</td>
</tr>
<tr>
<td>T259</td>
<td>0.009 ± 0.0002</td>
</tr>
<tr>
<td>T244</td>
<td>0.028 ± 0.004</td>
</tr>
<tr>
<td>T236</td>
<td>≤ 0.02</td>
</tr>
<tr>
<td>T131</td>
<td>≤ 0.02</td>
</tr>
</tbody>
</table>

### 4.5.4 SUMMARY

The possibility that high spontaneous mutation frequency of the transgene could be a consequence of gene inactivation by hypermethylation was tested in a number of transfectants that had lost the $hprt$ gene function. Pooled mutant colonies were treated with 5-azacytidine to induce phenotypic reversion. Inducible reversion would suggest that spontaneously arising 6-TG$^R$ mutants were the result of gene inactivation through DNA hypermethylation (discussed in 4.2.4).
Six of the 9 transfected clones demonstrated no spontaneous or 5-azacytidine inducible reversion of 6-TG\(^R\) mutants to HAT\(^R\), suggesting that "epigenetic factors" were not responsible for the high mutation rates observed in these transgenic cell lines. Some 6-TG\(^R\) mutants from clone T2411 were capable of reversion to HAT\(^R\) at a frequency of 6 x 10\(^{-5}\). These HAT\(^R\) colonies were not resistant to aminopterin alone, suggesting that the initial loss of \(hprt\) gene function in these mutants was a consequence of inactivation by methylation, followed by reactivation after 5-azacytidine treatment. Hypermethylation could be suggested as one mechanism for inactivation of the \(hprt\) minigene in clone T2411, which was shown to have a single transgene integration. However, it appears that hypermethylation did not play a major role in loss of gene function observed in the remaining 6 transfected clones. 6-TG\(^R\) mutants from 2 of the 9 transfectants (T131 and T238) were found to be aminopterin resistant. These clones were excluded from further studies.

After 5-azacytidine treatment, all pooled mutants demonstrated a decreased plating efficiency (0.5% and 5% at 1 and 3 \(\mu\)M concentrations respectively) when compared to survival of similarly treated parental TK6 cells (section 4.2.3.4, Figure IV.3). For reasons not known, \(hprt\) cells may have a depleted pool of intracellular cytosine. This could cause a competition for DNA incorporation between 5-azacytidine and cytosine resulting in a generalized demethylation possibly leading to increased sensitivity to 5-azacytidine.

Southern analysis of 5 or 6 mutant clones from each transgenic population supported the hypothesis that DNA changes were responsible for \(hprt\) transgene
inactivation. In a total of 40 informative mutant clones, 22 (55%) demonstrated a total loss of all \textit{hppt} transgene bands and 18 (45%) were a consequence of partial deletion. Small scale deletions or point mutations were not the cause of gene inactivation in any of the mutant clones tested, since the observed banding pattern for this type of mutation would likely have been identical to that of the parental cell line. A statistical simulation program (Appendix 3) was designed to test the probability of selecting mutant siblings for analysis. Data in Table IV.8 demonstrated that this probability was low, suggesting that the mutants tested were independent events.

From the evidence presented in this section, it was concluded that the a large-scale deletion, or complete loss of the transgenic sequences, was the major cause for the loss of \textit{hppt} gene function in transformed human lymphocytes. The observation that all of the spontaneous mutants recovered from the single-gene transfected clones (T164 and T2411) demonstrated only large scale mutations was unexpected (Figure IV.25a). The genomic instability of these two clones, is likely due to intrachromosomal recombination that is the consequence of the inherent instability of the region of integration or to instability induced in the region by the process of integration.
4.6 SECTION DISCUSSION

Modern medicine stands to benefit from molecular engineering research, such as the use of carrier viruses for vaccine production, the construction of transgenic animal models of human diseases and gene therapy. The successful development of these medical advances will depend upon the stable integration of transfected sequences. However, it has been repeatedly demonstrated that genomic instability predominates over stable integration which results in the steady decline of transgene expression.

A high frequency of transgene loss was reported by McBurney et al. (1994) and Schmidt-Kastner et al. (1996), who suggested that high genomic instability in mouse cells was due to recombinational loses of the transfected plasmid sequences. Work by Nickoloff et al. (1992) suggests that an increase in a gene's transcriptional activity could lead to enhanced intrachromosomal recombination and gene conversion involving direct repeats. It is possible that alleles harbouring tandem insertions demonstrate increased transcriptional activity, conditions that may result in the production of double strand breaks and subsequently in intrachromosomal recombination, an event which can cause deletions of the inserted sequences. A report by Köhler and Vogt (1994) showed evidence of spontaneous interstitial deletions of repetitive DNA sequences on human Y-chromosomes allowing maintenance of dicentric chromosomes. Also, Rainville et al. (1995) have suggested that non-random intragenic mutational deletions within the human hprt gene are likely a consequence of hairpin structure formation, within repetitive sequences, that is subsequently cleaved by topoisomerase II. A recent report by Collick et
al. (1996) describes findings similar to the observations made in this thesis. In their work, they demonstrate high instability of two different long inverted repeat transgenes in a transgenic mouse. They also suggest stabilization of the inverted repeat motif can occur by total transgene deletion or a deletion event resulting in the loss of the inverted repeat region. Their experiments support the hypothesis that single insertion transgenes are likely to be more stable than tandem repeats.

This chapter examines the fate of 9 different transflectant clones, which had integrated *hprt* genomic sequences during electroporation. Work by Meaking et al. (1995) and Vatteroni et al., (1993) suggested that certain electroporation conditions could induce damage in mammalian DNA, which in turn could be the potential site of biased transgene integration during the process of DNA repair (Kato et al., 1986; Rassool et al., 1991). Human *papilloma* virus (HPV-16) sequences have been found to preferentially integrate into *fragile sites* (Zimonjic et al., 1994). These *fragile sites* could be a consequence of unstable gene integration or, alternatively, the instability of the transfected sequences could be a consequence of the site of integration, suggesting that sites of integration may not be completely random.

The experimental evidence presented in this thesis suggests that the majority of integrations following introduction of linear DNA by electroporation consisted of multiple tandem insertions leading to unstable phenotype. Multiple gene copies, irrespective of the number of repeats, demonstrated increased genomic instability. The unique Southern analysis banding pattern of transflectants studied was suggestive of different sites of integrations, however, the genomic sequences at the site of integration
are as yet unknown.

The site of transgene integration and its subsequent stability may be cell type dependent. Telomerase activity has been reported in most tumour cells but not in normal somatic tissues (Kim et al., 1994). However, recent findings demonstrate that haemapoetic cells, both normal and malignant, show telomerase activity (Broccoli et al., 1995), and this activity may be up-regulated (Hiyama et al., 1995). Therefore, immortal lymphoblastoid TK6 cells used in this study potentially have telomerase activity (Broccoli et al., 1995). It is possible that the sticky ended hprt minigene is capped by telomeric sequences prior to integration into the genome. According to Murnane and Yu (1993), this capping of genomic sequences acts to protect the DNA from enzymatic degradation, allowing subsequent integration into chromosomal DNA. If this was the case, then the site of integration may be biased towards telomeric sequences by homologous integration (Farr et al., 1991). Short tandem telomeric repeats at the ends of transfected sequences could also explain the high frequency of transgene loss observed in these experiments possibly through recombinational events. Thus, the high rate of genomic instability observed in the TK6 transfectants may be enhanced by telomerase activity and might not be observed in transfected normal cells. Moreover, the site of integration of the transfected sequences could be associated with chromosomal break points, implying that DNA transfection, like ionizing radiation, can also induce generalized genomic instability and that the introduction of genomic sequences may be destabilizing factor. This contention is supported by the observations of Hastie and Allshire (1989) that telomeric sequences are hotspots for recombination, breakage, and fragility. Furthermore, Royle et
al. (1988), and Baird et al. (1995) have demonstrated that hypervariable microsatellites are clustered near the ends of chromosomes. In this regard, future research would be to investigate the chromosomal positions and genomic sequences surrounding the integrated \textit{hprt} minigene in the various clones discussed in this thesis. Characterization of the transgene integration sites could lead to better understanding of why even the most stable integrants are not as stable as endogenous genomic sequences.

It was observed that even the most stable transfectants in this study could not compete with the stability of the endogenous gene whose site of integration was established by evolution. A possible explanation offered by (Lima-de-Faria, 1980) is that positioning of all endogenous genes is optimized within each chromosomal field, and disruption of this optimized centromere-telomere arrangement may lead to genomic instability (Lima-de-Faria et al., 1991).

The transfected \textit{hprt} sequences used in this study were optimized for stable integration by ensuring that no bacterial or viral DNA sequences were present in the transgene. Foreign DNA sequences may activate cellular defence mechanisms. Previous reports of tandem insertions into \textit{Ascobolus immersus} were associated with DNA methylation reversible inactivation (MIP) (Rhounim et al., 1994), or methylation irreversible process (RIP) (Singer and Selker, 1995), both demonstrated to act in defence against repetitive sequences invading the host genome. Initially, these recently discovered mechanisms were thought to be functional only in premeiotic lower eucaryotes (Bowring and Catcheside, 1993). Recent publications have demonstrated gene quelling in both vegetative lower eucaryotes (Vijayaraghavan and Kapoor, 1996) and in
plants (Meyer and Saedler, 1996). Moreover, transgene silencing of *Neurospora*
vegetative cells has been demonstrated to be a dominant effect acting through *trans* RNA-DNA or RNA-RNA interactions (Evans et al., 1986b; Doetschman et al., 1988;
Kolchinsky and Gresshoff, 1993). Possibly a similar defence mechanism might also be
functional in higher eucaryotic cells. An investigation of the genomic stability of these
transfected sequences demonstrated strongest correlation between stable transgene
expression and single-gene insertions. These single gene insertions were almost as stable
as the endogenous sequences, but suggestive of a different mechanism of acquiring
spontaneous mutations. An unexplored possibility for the observed genomic instability of
the transfected genes could be the neighbouring sequences at the site of integration
(discussed above). However, if the integration site alone was governing transgene
stability, the expectation would be that single gene insertions would be just as susceptible
to inactivation as tandem repeats. This was not observed in experiments described in
section 4.4, where single gene insertions appeared to be more stable than tandem
insertions.

Attempts to correlate induced mutational events at the transgene locus and
different sites of transgene integration were unsuccessful for two reasons; 1) FISH
analysis using a 330 bp probe fragment (described in sections 2.5.5 and 2.6.2) failed to
detect the transgenes (data not shown) and ii) hypersensitivity to radiation of TK6 cells
and all derived clones reduced recovery of both HPRT and TK mutants, as seen earlier by
Amundson et al. (1993). Schwartz et al. (1995) demonstrated that the observed radiation
hypersensitivity was not a consequence of reduced capacity to rejoin double strand
breaks, but rather selective removal of the damaged cells by apoptosis (Schwartz et al., 1995). Apoptosis, characterized by cell shrinkage, chromosomal condensation, and endonuclease activation resulting in DNA cleavage, is a commonly observed event after irradiation of lymphocytes (Olive et al., 1993). TK6 cells (which have a wild type p53) appear even more sensitive to radiation than WTK1 cells (isolated from the same parental cell line and which express a mutant p53) (Little et al., 1995). Increased apoptosis, in response to radiation damage, is a likely explanation for the reduced mutation frequencies observed in Figures IV.23 and IV.24, a fact not widely appreciated at the start of this thesis. Irradiated cells may arrest in $G_2$, presumably allowing some cells to repair damage to DNA prior to initiating next cell division (Frankenberg-Schwager, 1989; Radford and Murphy, 1994). Mammalian cells exposed to caffeine prior to irradiation were observed to have a decrease in $G_2$ delay and enhanced DNA fragmentation (Rowley, 1992; Palayoor et al., 1995). However, the induced mutations at the transfected marker genes would be considered insignificant in comparison to the very high spontaneous mutation frequency observed.

From the observations made in this thesis it can be concluded that any newly developed transgenic cell line should be screened for single-gene insertions to ensure that stable gene expression is maintained.

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CHAPTER 5

5.1 CONCLUSIONS

The initial intent of this thesis was to establish a murine model system for detecting or identifying tumour factors that would contribute to genomic instability during tumour progression. The establishment of a mouse model capable of sensitively detecting mutational events, under both in vitro and in vivo conditions, should assist in the identification of the endogenous factors that contribute to tumour progression. This model system used a heterozygous marker to sensitively detect clastogenic events, results that might otherwise produce non-viable mutants at a hemizygous gene marker. This thesis demonstrates the success of the chosen cell line to detect both in vivo and in vitro mutational events. Preliminary studies presented implicate factors in tumour environment as possible contributors to accumulation of mutations and to tumour progression.

Evidence that mutational events were detected at different frequencies in different clones of the murine cell line led to an investigation of genomic instability of transfected sequences in a human cell line in the second part of the thesis. Construction of a number of TK6 derived clones provided a model to study the genomic instability of the transgenic sequences that presumably integrated at different chromosomal locations.

Multiple factors could affect stable transfection and expression of transgenes. These include the number of the integrated copies, the site of integration in the
chromosome, the chromatin structure and methylation state, and the strength and specificity of promoter elements. Sequences within the transgene that might function as recognition sequences for DNA-methylation or mRNA degradation may also contribute to stability of gene expression. Moreover, a transgene that was expressed in one cell line might be inactive in another genetic environment. Different mechanisms have been proposed to explain variegation of transgene expression: i) differential chromatin packaging (Grunstein, 1990; Hayes and Wolfe, 1992; Wallrath and Elgin, 1995); ii) DNA excision of transgenes (Kearns et al, 1995); iii) transgene localization to regions of the genome inaccessible to transcriptional machinery which may be compartmentalized within the nucleus (Henikoff, 1994).

At least two studies of transgene copy number and genomic instability (Robertson et al., 1995; Robertson et al., 1996) are consistent with the results reported in this thesis. In the 1995 paper, the high number of tandem insertions did not correlate with instability or decreased level of gene expression. However, none of the transgenic mice used in their study had a single gene insertion. The latter publication by this group (Robertson et al., 1996) further suggests that transgene expression in the mouse is age dependent and strongly correlated to transgene copy number. They demonstrate the tendency for transgene expression to decline with the age of the animal, and this decline was greatest in animals with the increased number of copies of the transgene. Their explanation is that the inactivation of the transgene is occurring by heterochromatization rather than loss of the transgene. However, their studies look for the presence of the transgene by methods that monitored the genotype of the majority of cells; thus, the sporadic losses of
transgenes in sub-population would not be detected.

The mosaic expression of transgenes has been reported in many studies (McBurney et al., 1994; Robertson et al., 1996; Festenstein et al., 1996; Schmidt-Kastner et al., 1996). It was suspected that this mosaicism or variegation of gene expression was associated with transgene integration into heterochromatin. Lima-de-Faria and Jaworska, (1968) define heterochromatin as usually late replicating regions of the chromosomes that remain condensed throughout the cell cycle and lack gene expression (Russell, 1963). It is possible some of the transfections described in this thesis went undetected as a result of integration into heterochromatic regions that were associated with gene inactivation, such as for the position effect variegation observed in Drosophila melanogaster (Henikoff, 1990). However, there is evidence to suggest that integration into heterochromatin does not necessarily result in gene inactivation (Bayne et al., 1994; Pardue et al., 1996). The gene for alpha-foetoprotein, for example, can remain active in the visceral yolk sac endoderm of the mouse even when integrated into the inactive heterochromatin region of the X chromosome (Krumlauf et al., 1986). Also, the expression of the hprt transgene integrated in a Y heterochromatic region of the transgenic mouse demonstrated mosaicism, suggesting that this region has only a relatively weak effect on transgene inactivation (Pravtcheva et al., 1994).

Using FISH to localize the λgt10 lacZ transgene in the Muta™ mouse, Blakey et al. (1995) showed that the integration occurred in a euchromatic G-C rich region of chromosome 3. These authors contend that the increased stability in this region may be due to a lower frequency of breaks observed within light staining bands (Yunis and
Soreng, 1984) when compared to the higher frequency occurring at the boundaries of euchromatic and heterochromatic regions.

From the observations made in this thesis, a single gene insertion appears to be the most stable arrangement for transgene integration into the genome. In the small number of transfectants studied in this series, a single copy integration occurred in 33% of transfectants. In establishing stably expressing transgenic lines, it may be useful to select clones that have a single-gene insertion.

Genomic instability is more evident in transfected sequences than in endogenous genomic sequences. However, it has also been demonstrated, using mouse fibrosarcoma cells, that endogenous sequences can also vary widely in their stability. For both endogenous or transfected genomic sequences, stability is strongly dependent upon location and multiplicity of the gene. For the first time, the evidence presented in this thesis suggests that the singularity of the transfected sequences is essential for transgene stability.
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VII


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XVIII


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## APPENDIX 1

### Evolution and Nomenclature of Mouse Fibrosarcoma Cells

<table>
<thead>
<tr>
<th>Evolution</th>
<th>Mouse Fibrosarc.</th>
<th>Tissue Culture Adapted</th>
<th>MNU/6-TG Induced Mutants</th>
<th>Revertants</th>
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<tr>
<td>Specific Nomenclature</td>
<td>MC1A</td>
<td>MC1A-C1</td>
<td>MC-TGR1 to MC-TGR17</td>
<td>MC-TGS17-1 to MC-TGS17-54</td>
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<td>General Nomenclature</td>
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<td>MC-TG₅</td>
<td>MC-TG₅</td>
<td>MC-TG₅</td>
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<tr>
<td>Phenotype</td>
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<td>HPRT⁺</td>
<td>HPRT⁺</td>
<td>HPRT⁺</td>
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<tr>
<td>Possible Genotype</td>
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<td>hprtₐₑ⁺ (multiple copies)</td>
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<td>hprtₐₑ⁺ or hprtₐₐ⁻ / hprtₐₐ⁻</td>
</tr>
<tr>
<td>Drug Selection</td>
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<td>HATᵣ</td>
<td>HAT₅</td>
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<td></td>
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### Evolution and Nomenclature of Mouse Myeloma Cells

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<td>HPRT⁺</td>
</tr>
<tr>
<td>Drug Selection</td>
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<td>HATᵣ</td>
</tr>
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<td>6-TGᵣ</td>
<td>6-TG₅</td>
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# Evolution and Nomenclature of Human Lymphoblastoid Cells

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<th>Transfectants</th>
<th>Revertants</th>
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<tbody>
<tr>
<td>Specific Nomenclature</td>
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<td>Not Done</td>
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<td>Total of 81 clones recovered</td>
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<td>Co60 γ-ray induced clones M50.1 *** M50.2 M300.1</td>
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<td>Total of 36 clones recovered</td>
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<td>Total of 110 clones recovered</td>
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<tr>
<th>Phenotype</th>
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<tr>
<td>Drug Selection</td>
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</tr>
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</table>

*** Clone transfected with hprt minigene
APPENDIX 2

Karyotype of MC1A-C1 and its derivatives

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Total Number of Chromosomes</th>
<th>Number of X Chromosomes</th>
<th>Number of Other Painted Chromosomes</th>
</tr>
</thead>
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<tr>
<td>MC1A-C1 (n=19)</td>
<td>71.3 ± 14.3</td>
<td>2.8 ± 0.6</td>
<td>3.4 ± 1.4</td>
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<td>MC-TGR17 (n=39)</td>
<td>65.6 ± 5.0</td>
<td>2.8 ±0.6</td>
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<tr>
<td>MC-TGR17-51 (n=20)</td>
<td>93.5 ± 11.1**</td>
<td>4.7 ± 0.9**</td>
<td>3.9 ± 1.1</td>
</tr>
</tbody>
</table>

Results represent mean ± standard deviation. The differences in number of total chromosomes and X chromosomes are both statistically significant when MC-TGR17-51 was compared with the other two strains (**p ≤ 0.001, ANOVA, Bonferroni post test) (Wilkinson et al., 1995).
APPENDIX 3

Notes on the "Hits.c" program

There are four key components of the program:
- the main routine (lines 28 to 87)
- procedure: nextgen (lines 90 to 134)
- procedure: sampling (lines 136 to 178)
- procedure: probsib (lines 203 to 219)

The other components mostly deal with housekeeping issues, such as parameter entry or data sorting. The main routine controls program flow and iterates through generations, simulation repeats and different datasets.

Broadly, the array "series" keeps all the information about the population. The zeroth member of the array (series[0]) has the count of the number of normal cells, those assumed identical at the start of the simulation. As the simulation proceeds and cells mutate, the variable "hits" contains the number mutated cells, and the array entries series[1] to series[hits] contain the numbers of cells of the different mutations. To go from one generation to the next (in "nextgen"), cells in series[0] to series[hits] are tested to see if there are further mutations: the result is a new value for "hits" and usually different entries in the "series" array. Periodically, a fraction of the cells of each type are removed (in "sampling"), with the effect that the counts in the series array is reduced and usually, the value of hits lowered as well.

nextgen
This procedure takes a distribution of cells and simulated mutations. Two approaches are used: the approach depending on the number of cells of the type under consideration (the criterion for this is held by the parameter "CUTOFF" set on line 9). For a small number of cells (e.g., under 1000) each cell is considered individually in lines 113 and 114. A uniform (0,1) random number is generated using an algorithm from Applied Statistics, (randwh: 1982, Vol 31, No 2, p 190) and a sufficiently small random number results in a simulated mutation or "hit" - line 114. For large numbers of cells, this is too time consuming and mutations in a random number of cells are simulated by using the normal approximation to the binomial distribution - lines 105-109.

sampling
A similar approach is adopted in the sampling of the cell population in the "sampling" procedure. For a small number of cells, a uniform random number is generated for each cell, and the cell is either retained or discarded - lines 158-159. For a large number cells, recourse is again made to the normal approximation to the binomial distribution - lines 147-151.
**probsib**
For a resultant distribution of different numbers of mutated cells in the array series[1] to series[hits] (note that series[0] is omitted), probsib returns the probability that any two cells randomly drawn from the pool of mutated cells, are of the same type of mutation. If there are, in total R mutated cells (*i.e.*, series[1]+series[2]+...+series[hits]=R) then it can be shown that the probability of a sibling is given by dividing the sum of C(series[i],2) by C(R,2) where C(n,m) is the combinatorial coefficient; *i.e.*, C(m,n) = m!(n-m)!/n! and where ! denotes a factorial.

**Observations**
This simulation was developed over a period of time and did not benefit from a planned design, implementation and testing. Ideally, a simulation such as this would be based on a massive distribution of potential cells in which lines of cells which are removed through *sampling* would be eliminated from the overall potential population. This approach reflects an appropriate statistical methodology in which an entire population frame is constructed and then sampling takes place within that frame. A concern with that approach here was limited time to develop the full model and limited computer resources: the entire population frame would have required extremely large computer resources.

In the *ad hoc* approach adopted here, we have proceeded chronologically in hopes of striking a reasonable balance between need for realistic, though not ideal results and resource limitations in terms of computing resources and program development time. It is believed that the difference between the population frame approach and this is minor.

“Hits.c” program

/* For Diana Wilkinson */
#include <stdlib.h>
#include <dos.h>
#include <math.h>
#include <stdio.h>
#include ".glib.h"

#define MAX_N_TYPES 15000
#define CUTOFF 1000

int xrand, yrand, zrand, ntypes;
unsigned long far series[MAX_N_TYPES], start_count, no_sims;
int n gens, harvest, generation, interim=0, output_dist=0, data_set;
float sample_frac, hit_prob;
FILE *in;

double randwh();
void getdata(void);
void dump_parameters(void);
void seeds(void);
void sampling(void);
int nextgen(void);
void sort(int n, unsigned long a[]);
void sorta(int n, unsigned long a[]);
int get_params(void);
float probsib(void);

main()
{
    extern int xrand, yrand, zrand;
    extern unsigned long series[MAX_N_TYPES];
    int i, j, k, err;

    seeds();
    // xrand = 125; yrand = 2314; zrand = 215;
    printf(\"nHTS\nSeeds: xrand=%d yrand=%d zrand=%d \n\n",xrand, yrand, zrand);
    if ((in=fopen("hits.dat","rt"))==NULL)
        { printf("Trouble opening data file hits.dat. in=%d \n\n",in);
          return(2);
        }

    data_set = 0;
    while ( get_params() != 0 )
    {
        data_set++;
        dump_parameters();

        /* Loop over several simulations ... */
        for (k=1; k<=no_sims; k++)
        {
            ntypes=1;
            series[0]=start_count;
            for (i=1;i<MAX_N_TYPES;i++) series[i]=0;
            for (generation=1; generation<=n gens; generation++)
            {
                if ( generation % harvest == 0 ) sampling();
            }
err = nextgen();
if (interim == 1) printf("Generation: %d, n(type0): %d, ntypes: %d\n", generation, series[0], ntypes);
if (err != 0)
{
  if (err == 1)
  {
    printf("*** Error condition: interim=%d and "ntypes); 
    printf("MAX_N_TYPES = %d\n", MAX_N_TYPES);
    return(1);
  }
  if (err == 2)
  {
    printf("*** Error condition Too many cells in the vessel! n=%d\n", series[0]);
    return(2);
  }
  if (generation != n_gens) for (j = 0; j < ntypes; j++) series[j] = 2 * series[j];
}
printf("Sim %d. Number of different types: %d, \%d, ntypes); 
printf("probability of a sib %d\n", probsi);

if (output_dist == 1 & ntypes > 1)
{
  printf("Distribution: ");
  for (i = 0; i < ntypes; i++) printf("%d, ", series[i]);
  printf("\n");
}
/* End of loop over repeated simulations ... */
} /* End of this set of parameters */
close(in);
return 0;

int nextgen(void)
{
    int i, j, new_hits, hits, ifault;
    double mean, stdv, ranx;

    /* At this point, it's deterministic ... will 
       add stochastic component for small counts later.
    */

    new_hits = 0;
    for (i = 0; i < ntypes; i++)
    {
        hits = 0;
        if (series[i] > CUTOFF) /* Deterministic if large enough */
        {
            mean = hit_prob * series[i];
            stdv = sqrt(mean * (1 - hit_prob));
            ranx = ppnd(randwh(), &ifault);
            ranx = mean + stdv * ranx;
            hits = ((ranx < 0) ? 0 : ranx);
        }
        else /* Otherwise stochastic */
        {
            for (j = 1; j < series[i]; j++)
                if (randwh() < hit_prob) hits++;
        }
    }
}

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if (hits > 0 )
{
    new_hits=new_hits+hits;
    series[i]=series[i]-hits;

    /* Got enough array space for new cell types? */
    if (ntypes+hits>MAX_NTYPES) { ntypes=ntypes+new_hits; return(1); }
    if (series[0] > 300000000) return(2);

    /* Now one new type for each */
    for (j=ntypes;j<ntypes+hits;j++) series[j]=1;
    /* Advance pointer to last element in array */
    ntypes=ntypes+hits;
    j=0;
}

return(0);

void sampling(void)
{
    int i, j, ifault;
    double mean, stdv, ranx;
    unsigned long count;

    for (i=0;i<ntypes;i++)
    {
        if (series[i] > CUTOFF ) /* Big count - deterministic */
            /* series[i] = sample_frac*series[i]; */
        {
            mean = sample_frac*series[i];
            stdv = sqrt(mean*(1-sample_frac));
            ranx = ppnd(randwh(),&ifault);
            ranx = mean + stdv*ranx;
            count = ((ranx < 0 ) ? 0 : ranx ) ;
        }
        else /* Small count - stochastic */

            {
                count = series[i];
                for (j=1;j<series[i];j++)
                    if ( randwh() > sample_frac ) count = count - 1;
                series[i] = count;
                count++;
            }

    /* Sort them so the zeros are at the end */
    // for (i=ntypes;i>=1;i--) series[i]=series[i-1];
    sorta(ntypes,series);
    // for (i=0;i<ntypes;i++) series[i]=series[ntypes-i];
    // series[ntypes] = 0;

    /* Now reset the number of types */
    i=0;
    while (i<ntypes)
{ if (series[i++]=0) break; }
        ntypes = i-1;
    if (interim==1) printf("After sampling, ntypes=%d\n",ntypes);
}

void dump_parameters(void)
{
    printf("\n\nMaximum array size %d\n",MAX_N_TYPES);
    printf(\"Data set number %d\n",data_set);
    printf("Number of simulations %d\n",no_sims);
    printf("Interim values: %d, output distribution: %d\n",
            interim,output_dist);
    printf("Number of generations %d\n",n_gens);
    printf("Sample every %d generations\n",harvest);
    printf("No. of cells to start with %d\n",start_count);
    printf("Probability of mutation %f\n",hit_prob);
    printf("Fraction of cells left after sample %f\n",sample_frac);
}

int get_params(void)
{
    if ((fscan(infile, "%d %d %d %f %f %d %d", &n_gens, &harvest, &start_count, 
        &sample_frac, &hit_prob, &no_sims, &interim, &output_dist) != 8)
        return(8);
    else
        { return(0); }
}

float prob_sim(void)
{
    unsigned long sum1, sum2, i, series;
    float probability;
    sum2 = 0; sum1 = 0;
    for (i=1;i<=ntypes;i++)
    {
        series = series[i];
        // if (seriesi <= 1 ) break;
        sum1 = sum1 + seriesi*(seriesi-1)/2;
        sum2 = sum2 + seriesi;
    }
    sum2 = sum2*(sum2-1)/2;
    if (sum2 == 0) sum2 = 1;
    probability = (float)sum1 / (float)sum2;
    return(probability);
}

void sort(int n, unsigned long ra[])
{
    // Heapsor algorithm from "Numerical Recipes in C" by

    int l, j, ir, i;
    unsigned long rra;

    if (n <= 1) return;
    l = (n >> 1) + 1;
ir = n;
for (i=0; i < ir; i++) {
    if (i > 1)
        ra[i] = ra[i-1];
    else
        ra[i] = ra[ir];
    ra[ir] = ra[1];
    if (-ir == 1) {
        ra[1] = rra;
        return;
    }
    i = 1;
    j = 1 << 1;
    while (j <= ir) {
        if (j < ir && ra[j] < ra[j+1]) ++j;
        if (rra < ra[j]) {
            ra[i] = ra[j];
            j += (i=j);
        }
        else j = ir + 1;
    }
    ra[i] = rra;
}

void sort(int n, unsigned long a[])
{
    int i, j;
    unsigned long temp;
    for (i=0; i<n; i++)
        for (j=i+1; j<n; j++)
            if (a[i] < a[j]) {
                temp = a[i];
                a[i] = a[j];
                a[j] = temp;
            }
}