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THE DEVELOPMENT OF A SENSITIVE HUMAN EPITHELIAL CELL ASSAY FOR ENVIRONMENTAL MUTAGENS

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THE DEVELOPMENT OF A SENSITIVE HUMAN EPITHELIAL CELL ASSAY FOR ENVIRONMENTAL MUTAGENS

By

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ABSTRACT

THE DEVELOPMENT OF A SENSITIVE HUMAN EPITHELIAL CELL ASSAY FOR ENVIRONMENTAL MUTAGENS

By

Yuh-Shan Jou

An ideal assay for environmental human mutagens should be: 1) relevant to human cell mutagenesis; 2) sensitive in detecting weak or low dose mutagens; 3) accurate in revealing mutational alterations at the molecular level, and 4) simple and inexpensive. Toward developing a cell line with these attributes, experiments were carried out to reactivate the hypoxanthine-guanine phosphoribosyltransferase (hprt) gene on an inactive X-chromosome by 5-azacytidine treatment in a 6-thioguanine-resistant (6-TG^r) human teratocarcinoma cell line, HTX6TG-1 [46,XX,t(15;20)], which appears to contain an X-ray deleted hprt gene on the active X-chromosome. One HAT^r clone (hprt-proficient), HTX6TG-1/5AC4-1 [46,XX,t(15;20) and t(11;X)], with low reversion frequency was selected from these

experiments for mutation studies. The results indicate that mutation frequencies induced by X-rays, UV, MNNG and daunomycin were strikingly increased in this cell line as compared to its parental cell line (5-50 fold increase). Molecular characterizations of X-ray-induced 6-TG^r mutants were performed with Southern blotting and hybridization with labeled hprt cDNA. An analysis of the autoradiographic banding patterns of hprt genes shows that 72% (21/29) of 6-TG^r mutants induced by X-rays are deletion mutants, confirming previous reports that X-rays induced mostly deletion mutations. The results also indicate that the HTX6TG-1/5AC4-1 cell line contains an intact hprt gene and a deleted hprt gene encompassing the 7 to 9 exons. Furthermore, experiments were carried out to determine whether the mutagen-sensitive cell line can be used to reveal the nature of mutation by direct nucleotide sequencing of polymerase chain reaction (PCR) amplified hprt cDNA. The results indicate that the cell line is useful for this purpose. An analysis of six independently derived UV-induced 6-TG^r mutants shows that most of the mutations (50%) involved T·A to A·T transversions instead of G·C to A·T transitions which were prevalent in previous studies. All these mutations are attributable to misincorporation of bases opposing dipyrimidines during the DNA replication. This new assay appears to have many desirable features of an ideal mutation assay and should be useful for assessing the mutagenic potential of various environmental agents.

To:

My Wife, Ruey-Hwa Chen

My Parents, My Brothers And My Sister

For Their Love And Support

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5AC	5-Azacytidine
6-TG^r	6-Thioguanine resistant
8-AG	8-Azaguanine
aprt	Adenine phosphoribosyltransferase
Brdu	5-Bromodeoxyuridine
CHO	Chinese hamster ovary
DEPC	Diethylpyrocarbonate
dhfr	Dihydrofolate reductase
DMSO	Dimethylsulfoxide
DTT	Dithiothreitol
EPA	Environmental Protection Agency
FIFRA	Federal Insecticide, Fungicide and Rodenticide Act
gpt	Xanthine-guanine phosphoribosyltransferase
HAT^r	Hypoxanthine, aminopterin, and thymidine resistant
HDL	High dose level
hprt	Hypoxanthine guanine phosphoribosyltransferase
IARS	International agency for research on cancer
M-MLV	Moloney murine leukemia virus
MNNG	N-methyl-N'-nitro-N-nitrosoguanidine
MTD	Maximum tolerated dose
NAS	National Academy Science
NCI	National Cancer Institute

NTP	National Toxicology Program
OTA	Office of Technology Assessment
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PRPP	5'-phosphoribosyl-1-pyrophosphate
SCE	Sister chromatid exchange
SSC	Sodium chloride sodium citrate
SSPE	Sodium chloride sodium phosphate EDTA
TBE	Tris-borate EDTA
TEMED	N,N,N'N'-Tetramethylethylenediamine
TFT	Trifluorothymidine
TK	Thymidine kinase
TSCA	Toxic Substances Control Act
UDS	Unscheduled DNA synthesis
UV	Ultraviolet light

INTRODUCTION

Mutation is a major source of genetic variation. On one hand, it is required for the evolution and adaptation of a species. On the other hand, it is the source of genetic diseases when mutations occur in germ cells and somatic diseases such as cancer and atherosclerosis when mutations occur in somatic cells. As human beings intervene with modern technology to eliminate major perilous elements in natural environment, acute infectious diseases take fewer human lives, more human beings live longer and are manifesting chronic diseases (e.g., cancer and cardiovascular disease) in part due to longer exposure to natural mutagens/carcinogens and environmental pollutants (Trosko and Chang 1978).

It is estimated that there are at least 60,000 existing chemicals in commerce and industry, and about 1,000 new chemicals are introduced each year (NAS 1984). These chemicals do not include most naturally-occurring products or pesticides that human beings ingest in food and beverages. Of the natural pesticides that have been tested in at least one rodent species, about 50 percent (27/52) are found to be rodent carcinogens (Ames et al. 1990). Approximately 1,000 chemicals have been tested in some forms of the rodent cancer bioassay. As a result of these studies, about 600 of them are

labeled as rodent carcinogens, 100 of them are non-carcinogens, and the remaining 300 are inconclusive (Gold et al. 1984, 1986, 1987). These alarming figures, and the non-genotoxicity in short-term tests of many potent carcinogens tested in the National Toxicology Program (Tennant et al. 1987), cast doubt about the validity of these tests and their relevance to human exposure, especially the use of maximum tolerated dose (MTD) of chemicals in in vivo and in vitro assays for mutagens/carcinogens (Ames and Gold 1990a). The controversy also calls for the development of reliable, sensitive, and relevant assays for human enviromental mutagens/carcinogens.

An ideal assay for human enviromental mutagens should be: 1) relevant to human cell mutagenesis, 2) sensitive in detecting weak or low dose mutagens, 3) accurate in revealing mutational alterations at the molecular level, and 4) simple and inexpensive. Although it may not be possible to have all of these attributes in a mutation assay, an assay system with several of these features will be preferred.

Toward developing an in vitro mutation assay with several of these attributes, I have embarked on improving the sensitivity of a human cell mutation assay based on the following hypothesis. At present, the mutation assay at the hypoxanthine guanine phosphoribosyltransferase locus (hprt) is the best and most commonly used mutation assay in mammalian including human cells. Owing to the location of this gene on a single functional X-chromosome, many induced mutants may not

be viable if these mutants also contain deletions or mutations of genes essential for cell survival on the same chromosome. Therefore, by deleting this gene on the active X-chromosome and by using a reactivated hprt gene, on the non-essential inactive X-chromosome, as target gene for mutations, the sensitivity of the assay will be enhanced, since mutants with deletions or mutations of other genes on the same chromosome will be viable. Indeed, higher induced mutant frequencies have been reported in rodent cell lines with target genes on a non-essential chromosome (Waldren et al. 1986) or on one of the two functional homologous chromosomes (see review by DeMarini et al. 1989). A human epithelial cell line with near diploid karyotype [46,XX,t(15;20) and t(11;X)] developed according to this strategy by this dissertation research was, indeed, found to be more sensitive to various physical and chemical mutagens.

In addition, experiments were carried out to determine if this mutagen-sensitive cell line can be used to reveal the molecular nature of mutations by direct nucleotide sequencing of polymerase chain reaction (PCR) amplified hprt cDNA (Yang et al. 1989). The results of this study on UV-induced 6-thioguanine-resistant mutants (hprt-deficient) indicate that this cell line is useful for this purpose.

Therefore, this new mutation assay clearly has many of the desirable features of an ideal mutation assay mentioned previously and should be useful for assessing the mutagenic potential of various environmental agents. The development

and characterization of this cell line is detailed in this thesis.

LITERATURE REVIEW

The development of a new sensitive and accurate mutation assay using human epithelial cells is the focus of this dissertation research. Literatures in the following three areas will be reviewed: (I) mutations and human diseases; (II) assay systems for environmental mutagens/carcinogens; and (III) molecular analyses of mutations.

I. Mutations and Human Diseases

Mutations are hereditable changes in the genetic information on either the DNA or chromosomal level. Mutations in germinal cells may result in heritable genetic disease or birth defect (Epstein et al. 1983). Many germinal mutations are the result of point mutations leading to metabolic disorders such as phenylketonuria, galactosemia, Lesch-Nyhan syndrome, thalassemia, Tay-Sachs disease, etc. (McKusick 1986, Cotran et al. 1989). At the chromosomal level, germ line mutations may result in birth defects and chromosomal diseases such as Down's syndrome (trisomy 21) (Lejeune et al. 1959) and Turner's Syndrome (46, XO) (Ford et al. 1969). Some germinal mutations may affect DNA repair and chromosomal integrity such as xeroderma pigmentosum (reviewed by Cleaver 1990), Bloom's syndrome (Warren et al. 1981, Vijayalaxmi et al. 1983),

Fanconi's anemia (Schwaiger et al. 1982), ataxia telangiactasia (reviewed by Boder 1985), and Fragile X syndrome (Sutherland 1983) resulting in mutations and chromosomal aberrations in affected individuals. On the other hand, mutations in somatic cells have been postulated to play a major role in many somatic diseases (Lower and Kanarek 1982), Cancer (Boveri 1914, Knudson 1973), teratogenesis (Saxen 1976), atherosclerosis (Benditt 1977), senile cataracts (Hartman 1983), and aging (Failla 1958, Szilard 1954, Comfort 1964, Sinex 1974, Trosko and Hart 1976).

Among these, cancer apparently is the most important human disease whose etiology has been clearly shown, in some cases, to be related to mutagenesis. Evidence supporting the mutation theory of cancer are as follows: 1) Many carcinogens are mutagens (McCann and Ames 1975); 2) Tumor cells have a clonal origin (Linden and Gartler 1965, Manchalonis and Nossal 1968, Fialkow 1974, Nowell 1976, Taguchi et al. 1984); 3) Certain human syndromes that predispose the individual to cancer (e.g., xeroderma pigmentosum, Bloom's syndrome) have higher mutation rates of induced or spontaneous mutations (Maher and McCormick 1976, Glover et al. 1979, Warren et al. 1981, Vijayalaxmi et al. 1983); 4) Hereditary Wilm's and retinoblastoma tumors occur bilaterally more frequently and appear earlier than non-hereditary tumors of the same tissue (Knudson 1975); 5) The monomerization of UV-induced pyrimidine dimers by photoreactivation is correlated with the reduction of UV-induced tumors in *Poecilla formisa* after photoreactivation (Hart and Setlow 1975); 6) Gene or

chromosomal mutations affecting oncogenes (genes causing cancers) or tumor suppressor genes in somatic cells lead to tumor development [e.g., point mutations in ras (Bos et al. 1989) and p53 (Hollstein et al. 1991); translocations involving c-myc in Burkitt's lymphoma (Zech et al. 1976) and bcr/abl in chronic myeloid leukemia (Nowell and Hungerfold 1960); amplifications of N-myc in neuroblastoma (Schwab et al. 1983) and c-erb B2 in breast cancer (Slamon et al. 1987); and loss of heterozygosity in colorectal (Fearon and Vogelstein 1990) and breast cancers (reviewed by McGuire and Maylor 1989)]; and 7) Germ line p53 mutations in the familial Li-Fraumeni syndrome predispose the affected individuals to breast cancer, sarcomas and other neoplasms (Malkin et al. 1990).

Carcinogenesis is a multi-mechanism, multistage process involving three major processes: initiation, promotion, and progression (Reddy et al. 1982). Experimental evidence indicates that tumor initiators are mutagens whereas tumor promoters are mitogens that clonally amplify an initiated cell (Cohen and Ellwein 1990). Furthermore, tumor progression was accomplished by initiators but not by promoters (Hennings et al. 1983).

The genetic mechanisms involved in carcinogenesis have been documented (Table 1). These mechanisms include point mutation, chromosomal deletion, chromosomal translocation, aneuploidy, polyploidization, gene amplification, insertional mutagenesis, mitotic crossing-over and loss of heterozygosity

(due to mutations, deletions, mitotic crossing-over or loss of chromosomes).

Table 1. Genetic changes in human tumor cells

Type of mutation	Example	Reference
Gene mutation	Human bladder tumor. G to T transversion in codon 12 of c-Ha-ras	Reddy et al. 1982
Chromosomal translocation	Burkitt's lymphoma, t(8;14), involving transposition of c-myc	Zech et al. 1976
Chromosomal deletion	Retinoblastoma, inherited deletion of chromosome 13q14	Yunis & Ramsay 1978, Cavenee et al. 1983
Aneuploidy	Chronic lymphocytic leukaemia, trisomy 12	Gahrton et al. 1980
Gene amplification	Neuroblastoma, 5-1000 X amplification of N-myc	Schwab et al. 1983
Insertional mutagenesis	Embryonal carcinoma, insertional mutagenesis by retroviruses	King et al. 1985
Mitotic recombination	Wilms tumor, mitotic recombination occurs between the parathyroid hormone locus and 11p13	Raizis et al. 1985
Loss of heterozygosity	Retinoblastoma, expression of recessive alleles by chromosomal mechanisms	Cavenee et al. 1983
Polyploidy	Many tumors are hyperploid in karyotypes e.g. HeLa, MCF-7	Gey et al. 1952, Soule et al. 1973

II. Assay Systems for Environmental Mutagens/Carcinogens

Two major systems, *in vivo* rodent lifetime bioassays and *in vitro* short-term genotoxic tests, have been developed in the past to predict the mutagenicity/carcinogenicity for chemical hazards. Literature in these areas, especially the mammalian cell mutation assays are reviewed.

A. In vivo rodent lifetime bioassays:

The standard animal carcinogen bioassay designs adopted by different regulatory agencies are listed in Table 2. Scientists and regulators have reservations about using the lifetime rodent bioassays as the "gold standard" for predicting human carcinogenicity but no generally accepted alternative exists (Clayson 1987, Ames et al. 1987). Several principles and assumptions for risk assessment of effects of long-term exposure to carcinogens by using rodents bioassays have been established by the risk assessment community (NAS 1977 and 1986): (1) Effects in animals, properly qualified, are applicable to humans. (2) Methods do not now exist to establish a threshold for long-term effects for toxic agents. (3) The exposure of experimental animals to toxic agents in high doses is a necessary and valid method of discovering possible carcinogenic hazards in humans. (4) Agents should be assessed in terms of human risk, rather than as "safe" or "unsafe." A standard animal carcinogen lifetime bioassay includes carcinogens testing in two inbred rodent species, mice and rats. First, potential carcinogen should be used to

Table 2. Standard Animal Carcinogen Bioassay Designs

	NCI	FIFRA	TSCA
Species	Rats & mice	Rats & mice	Rats & mice
No. animals (M/F) at each dose	50/50	50/50	50/50
No. of dosages	2: MTD 1/2 or 1/4 MTD control (0 dose)	3: MTD 1/2 or 1/4 MTD 1/4 or 1/8 MTD control (0 dose)	3: HDL 1/2 or 1/4 HDL 1/4 or 1/10 HDL control (0 dose)
Dosing regimen			
Start	6 weeks	In utero or 6 weeks	6 weeks
End	24 months	Mice: 18-24 months Rats: 24-30 months	24-30 months
Observation period:	3-6 months after end of dosing	NS	NS
Organs and tissues to be examined	All animals: external and histopathologic exam (ca. 30 organs and tissues)	All animals: external exam; some animals pathological exam of 30 organs and tissues, other animals; fewer organs and tissues	All animals: external and histopathologic exam of (ca. 30 organs and tissues)

Abbreviations: NCI, National Cancer Institute;
FIFRA, Federal Insecticide, Fungicide and Rodenticide Act;
TSCA, Toxic Substances Control Act;
MTD, Maximum tolerated dose, causes minor acute toxicity;
HDL, high dose level, causes some acute toxicity;
NS, not specified.
Source: From OTA 1981.

determine the Maximum Tolerated Dose (MTD) which is the highest dose of potential carcinogen that causes minor acute toxicity without affecting the normal lifespan of animals. Generally, the MTD is subdivided to one half, one quarter or even one eighth of doses. These doses plus the MTD and no dose control are individually administered to 50 female and 50 male animals everyday for two years. At the end of the experiments, all animals are sacrificed to examine any external or histopathological toxicity or cancer for 30 organs and tissues. According to these standard experimental designs and principles, long-term rodent bioasseys have identified as carcinogens about 65% of 800 chemicals tested (Gold et al. 1984 and 1986). However, tests for human carcinogens using lifetime rodent bioassays are expensive, time-consuming and cost-ineffective (Lave et al. 1988). The standard rodent biosassays require the determination of the Maximum Tolerated Dose (MTD) of an agent, an excellent animal breeding facility and reliable histopathological examinations of rodent tissues. It has been estimated that direct costs of a rodent bioassay is one million per chemical (Lave et al. 1988). From the results of 100 chemicals tested by the US National Toxicology Program and reviewed by several researchers (Lave et al. 1988), the comparison between mouse and rat are 70% concordance (agreement of test outcomes in both rodent species), 70% sensitivity (proportion of rat non-carcinogens that are negative in mice) and 65% accuracy of rodent carcinogens (ratio of all positive test outcomes to all

chemicals tested). However, it is generally agreed that rats and mice are more similar biologically to each other than either is to humans. Therefore, the 70% concordance in rodents appears to be an upper bound for rodent-human concordance. According to the current policy, we need to assume that 65 out of 100 chemicals tested in rodents are human carcinogens and 70% of the chemicals showing concordance between rodents are able to be extrapolated to humans. In addition, these researchers predict 50 of the 65 are human carcinogens (77% sensitivity) and 20 of the 35 are not human carcinogens (57% specificity). It is even more ambiguous that only 12% of predicted 50 human carcinogens were positive in systematic Salmonella mutagenicity testing of 2,205 chemicals in Japan (Matsushima 1987).

B. In vitro short-term tests

It is obvious that in vitro short-term tests have the advantages of conducting relatively quick and inexpensive assessment compared to long-term rodent bioassays without involving testing in animals. In recent decades, several short-term genotoxic tests from prokaryote and mammalian cells have been used for risk assessment. These effects can be evaluated experimentally by measuring two categories of genetic effects : (1) gene mutation on specific-locus, such as the Salmonella/microsome mutagenesis assay (the Ames test) (Ames et al. 1973a, Haworth et al. 1983), mammalian cell (CHO or Chinese hamster V79) mutation assay at the hprt locus (Hsie

et al. 1981), and the mouse lymphoma L5178Y $TK^{+/-}$ cell mutation assay (Clive et al. 1972, Evans et al. 1986). (2) Chromosomal aberration assays including chromosome aberrations assay in CHO cells (Galloway et al. 1985), sister chromatid exchange (SCE) in CHO cells (Carrano et al. 1978, Galloway et al. 1985) and the micronucleus assay (Schmid 1975).

1. Salmonella/microsome test (the Ames test)

The Salmonella/microsome test (the Ames test) is the most popular short-term genotoxic assay and has been validated by more than 5,000 chemicals (Maron and Ames 1983, Kier et al. 1986). The Ames test measures reverse mutation from histine auxotrophy to prototrophy in several specially constructed mutants of Salmonella typhimurium to detect as many classes of mutagens as possible. A mutation (rfa) with modified cell wall to allow the entry of large molecules, such as polycyclic hydrocarbons, aromatic amines and aflatoxins, has been constructed. In addition, an excision repair deficient (UvrB) mutation and a plasmid pKM101 carrying an error-prone repair gene (mucAB) have been introduced to the bacteria to maximize the sensitivity of the mutation assay. The use of a rat liver homogenate (also called S9 fraction) to the bacterial suspension provides an approximation of mammalian metabolism to convert potential mutagens to their active metabolites (Ames et al. 1973b). Early studies of concordance between results from the Ames test and rodent carcinogenicity tests were reported with 90% or better sensitivities (percentages of

carcinogens identified as mutagens) and specificities (percentages of noncarcinogens identified as noncarcinogens) (McCann et al. 1975). However, the accuracy of their prediction for carcinogenicity is in doubt (Trosko 1988). Two recent studies have indicated that the predictability of a mutagenic response for rodent carcinogenicity has 75% and 85% sensitivities; 48% and 51% specificity (Tennant et al. 1987, Zeiger and Tennant 1986).

2. Mammalian cells for short-term mutation assays

2a. CHO/HPRT assay

The use of mammalian cells for mutagenesis research and genotoxic assays was possible after the development of techniques for growing mammalian cells in culture, in particular the measurement of cell survival by quantitating the colony formation from individual cells (Puck et al 1956). These techniques enabled the study of specific-locus mutation of the hypoxanthine-guanine phosphoribosyltransferase (hprt) gene in cultured Chinese hamster V79 cells (Chu and Malling 1968).

Hypoxanthine-guanine phosphoribosyltransferase is a purine salvage enzyme which converts hypoxanthine and guanine to their respective nucleotides, IMP and GMP, thus providing a salvage pathway for the synthesis of purine nucleotides (Figure 1). For somatic cell geneticists, there are several reasons for using the hprt system to assay compounds for mutagenic activity: (i) the hprt gene is one of the most

Figure 1. Purine biosynthesis, regulation, interconversion, and catabolic pathway. APRT: adenine phosphoribosyl-transferase (E.C. 2.4. 2.7); HGPRT: Hypoxanthine-guanine phosphoribosyltransferase (E.C. 2. 4. 2.8.); A: adenine; AR: adenosine; AMP: adenosine 5'-monophosphate; G: guanine; GR:guanosine; GMP: guanosine 5'-monophosphate; H: hypoxanthine; HR: inosine; IMP: inosine 5'-monophosphate; PRPP: 5'-phosphoribosyl-1-pyrophosphate; sAMP: adenylsuccinic acid; X: xanthine; XR: xanthosine; XMP: xanthosine 5'-monophosphate; 2,6-DAP: 2,6-diaminopurine; 2-FA: 2-fluoroadenine; 8-AH: 8-azahypoxanthine; 6-MP:6-mercaptopurine; 8-AG: 8-azaguanine; 6-TG: 6-thioguanine. Dotted arrows indicate feedback inhibition. (from Chu and Powell 1976)

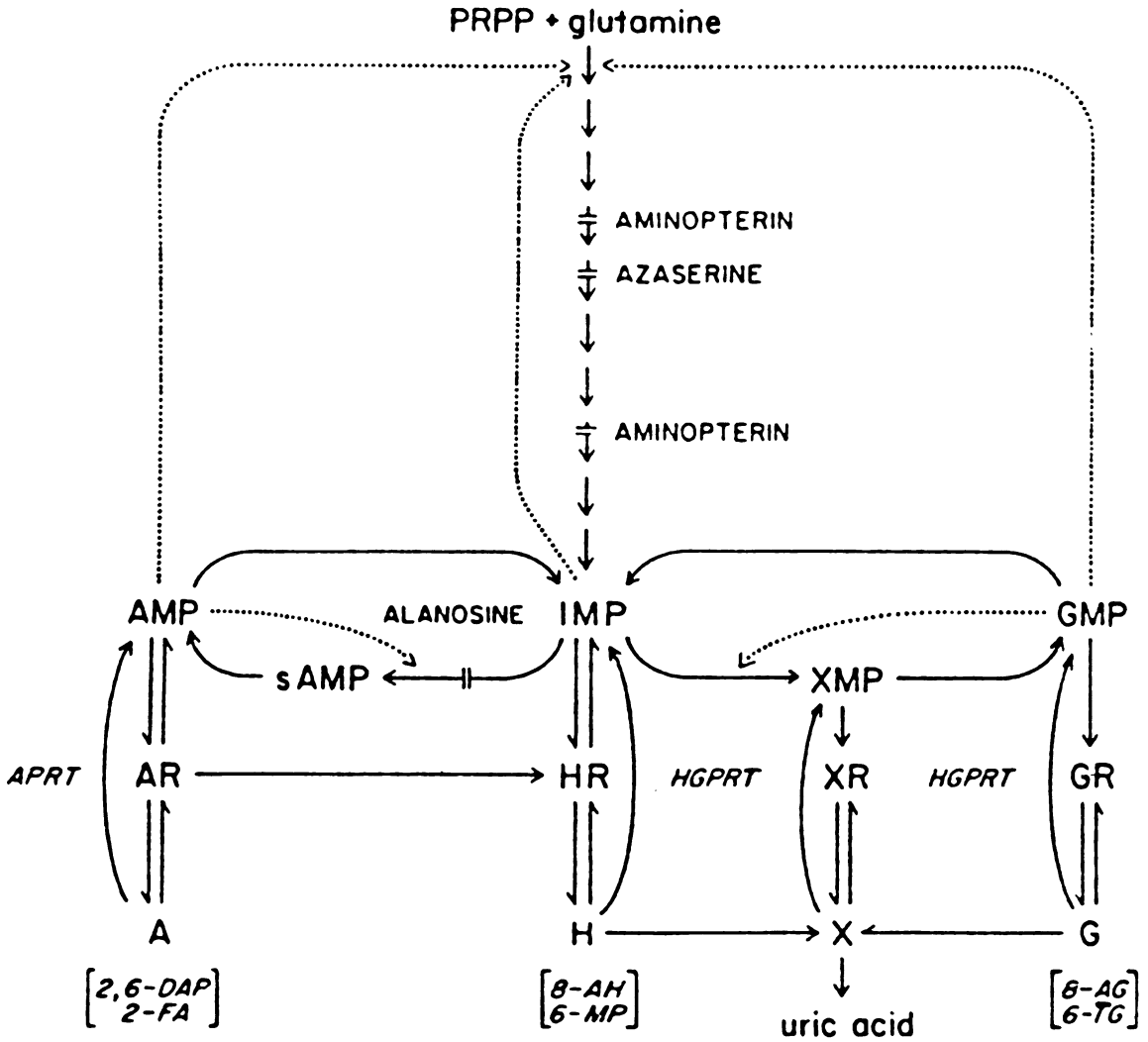

PRPP + glutamine
AMINOPTERIN
AZASERINE
AMINOPTERIN
AMP
ALANOSINE
IMP
GMP
sAMP
XMP
APRT
AR
HR
HGPRT
XR
HGPRT
GR
A
H
X
G
[2,6-DAP 2-FA]
[8-AH 6-MP]
uric acid
[8-AG 6-TG]

Figure 1

intensively studied genes in cultured mammalian cells; (ii) it is relatively easy to select for or against hprt gene expression in mammalian cells; (iii) the mutagenic effect on the hprt gene is dose-dependent for several classes of mutagens such as X-rays, ethylmethanesulfonate (EMS) and ultraviolet light.

Since the hprt gene is located on the X-chromosome (Xq26-27) (Pai et al. 1980), a single mutation can create a hprt-deficient mutant ($hprt^-$) because the hprt gene is functionally hemizygous in all mammalian cells. Females have only one functional X chromosome while the other X chromosome is inactivated by hypermethylation. HAT medium containing hypoxanthine (a purine source), aminopterin (an inhibitor of purine and thymidine synthesis) and thymidine (a pyrimidine source) has been used to kill $hprt^-$ cells which were rendered purine-deficient. However, hprt deficient mutants can be selected by growing in a medium containing 6-thioguanine (6-TG) or 8-azaguanine (8-AG) (Szybalski 1958). These purine analogues are converted to toxic nucleotides by the hprt enzyme, thus killing the wild type ($hprt^+$) cells. Cells lacking the hprt enzyme activity are unable to metabolize these purine analogues and thus survive in the selection medium.

The CHO/HPRT genotoxic assay was characterized by Hsie et al. (1975) and standardized into a quantitative assay for mutagenicity testing by Li et al. (1987). The performance of the CHO/HPRT genotoxic assay with 121 chemicals belongs to 25

chemical classes published from mid-1979 through June 1986 was reviewed and evaluated by the US Environmental Protection Agency Gene-Tox Program (Li et al. 1988). The evaluations by investigators have concluded that a good correlation exists between the mutagenic activity of chemicals in the CHO/HPRT assay and animal carcinogenicity results and recommended its inclusion in any assay battery. Additional studies have indicated that only 5-10% of X-ray induced $hprt^-$ mutants contain visible aberrations of the X chromosome (Thacker and Cox 1984, Muir et al. 1988). The extensive cell killing with the treatment of clastogens (chemicals that induce structural chromosomal abberrations) or radiation can produce serious errors in quantitative assessment of mutations of the hprt locus on a functionally hemizygous X chromosome (see reviews in the following section , 2b and 2c).

2b. Mouse lymphoma L5178Y/$TK^{+/-}$ assay

Another popular mutation assay utilizes the thymidine kinase (tk) gene which is located on the autosomal chromosome 17q21-22 (Kit 1976) in human cells and chromosome 11 in the mouse (Evans et al. 1986). TK is an enzyme in the pyrimidine salvage pathway that catalyzes the phosphorylation of thymidine to dTMP. HAT medium is doubly selective for both hprt and tk gene loci owing to the blockage of de novo synthesis of purines and pyrimidines. TK-deficient mutants are selected from mutagen-treated $tk^{+/-}$ cells with trifluorothymidine (TFT) or 5-bromodeoxyuridine (BrdU) (Clive

et al. 1979). A mouse strain of L5178Y lymphoma cells with a heterozygous autosomal $tk^{+/-}$ locus was developed to select for specific-locus mutations and chromosomal mutations (Clive et al. 1972). The assay has a unique advantage in its ability to differentiate two types of mutations, i.e. point mutations indicated by large colonies and chromosomal mutations indicated by small colonies. As illustrated in Figure 2, since hprt is a hemizygous nonessential gene on the X chromosome, viable mutants can be recovered with single gene mutations such as base substitutions, frameshifts, intragenic deletions and intergenic deletions without any effect on adjacent essential genes. However, large deletions or other chromosomal mutations extending into neighboring essential genes could be lethal to mutants. In contrast, the tk is a heterozygous autosomal gene. Mutants with multilocus deletion or chromosomal mutations will be viable because the functions of neighboring essential genes if deleted or mutated on one chromosome are provided by genes on the homologous chromosome. DeMarini et al. (1989) reported that weak mutagens such as UV induced similar frequencies of mutants in both the hprt locus and the tk locus. However, the viable mutants from the L5178Y/$TK^{+/-}$ mutation assay have 2-10 fold increase than that from the CHO/HPRT mutation assay after the treatment of clastogens or chromosomal mutagens (Figure 3). Several studies using the L5178Y/$TK^{+/-}$ genotoxic assay reported that there is a 70% concordance (agreement of test outcomes in both rodent species), sensitivity (proportion of rat carcinogens that are

Figure 2. Recovery of mutants with gene or chromosomal mutations at heterozygous and hemizygous loci. Mutants with genetic damages confined within the target gene (gene mutation) are recoverable at either type of locus. Mutants with genetic damages involving the target gene and adjacent essential genes (e.g. chromosomal mutation) are lethal if the essential genes are on hemizygous regions. However, such mutants are recoverable if these essential genes are heterozygous beccause the deleted essential function is supplied by homologous region on the homologous chromosome . (from DeMarini et al. 1989)

Recovery of Mutants at Heterozygous and Hemizygous Loci

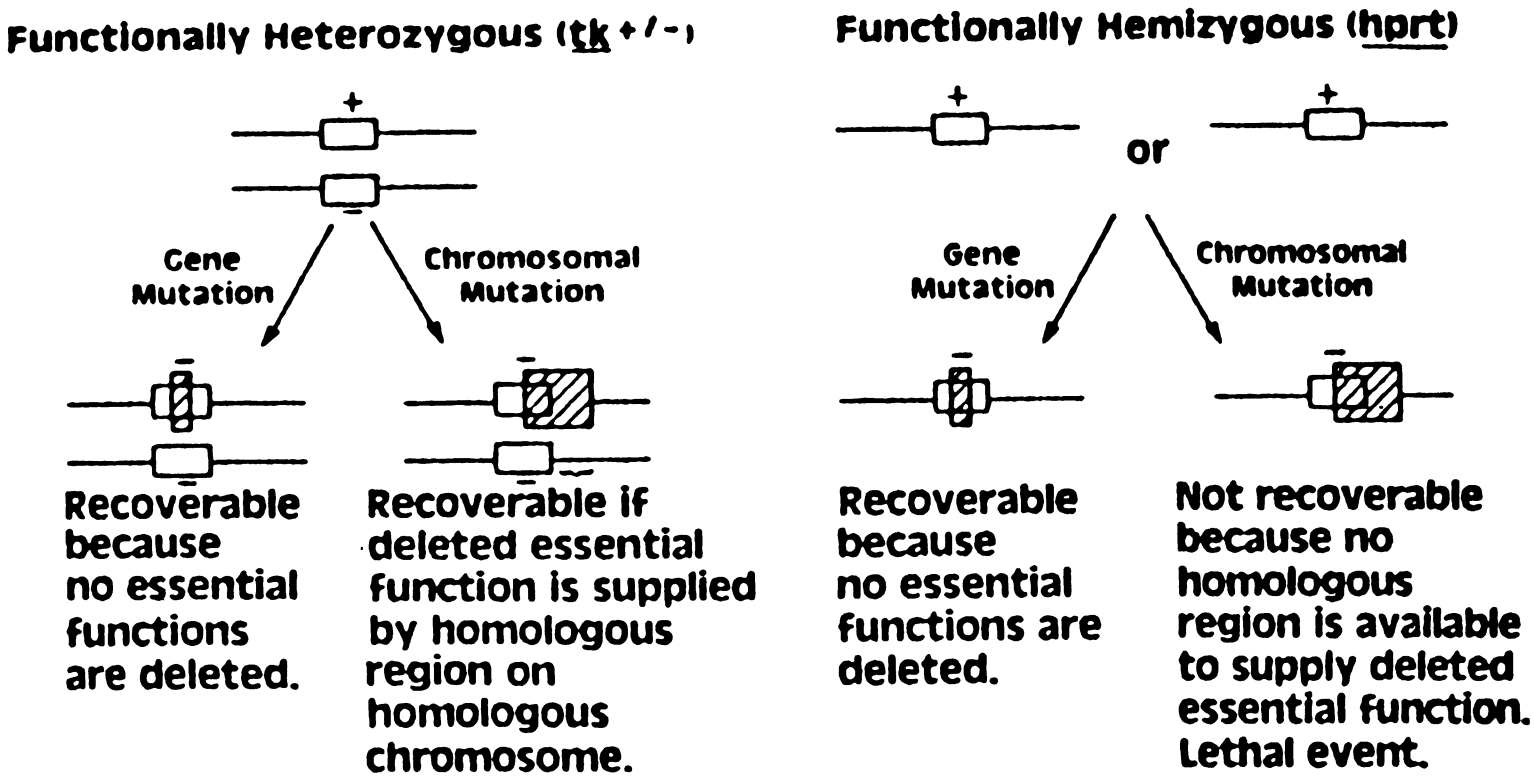


Figure 2

Figure 3. Induced mutant frequencies in two CHO and three L5178Y cell lines at 50% survival (calculated from linear regressions) by physical and chemical mutagens that produce primarily chromosomal mutations (ionizing radiation and hydrogen peroxide) or primarily gene mutations (UV light and EMS). (from DeMarini et al. 1989)

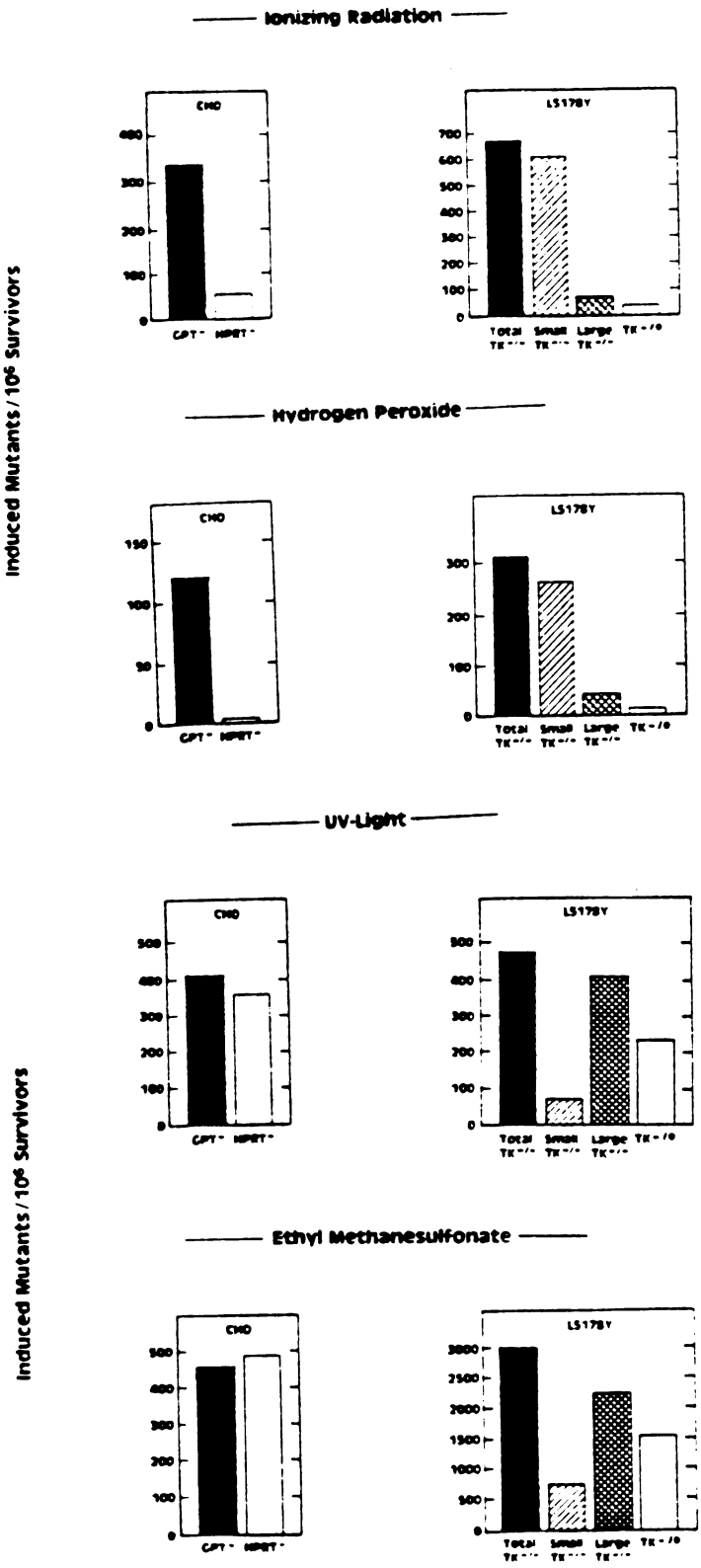

Ionizing Radiation
CHO
L5178Y
Hydrogen Peroxide
CHO
L5178Y
UV-Light
CHO
L5178Y
Ethyl Methanesulfonate
CHO
L5178Y
Induced Mutants / 10⁶ Survivors
Induced Mutants / 10⁶ Survivors

Figure 3

positive in mice) and specificity (proportion of rat non-carcinogens that are negative in mice) compared with rodent bioassays (Tennant et al. 1987, Zeiger et al. 1990, Myhr et al. 1990).

2c. pSV2gpt-transformed CHO cells (AS52) assay

Recently a pSV2gpt-transformed Chinese hamster ovary (CHO) cell line was developed (Tindall et al. 1984). The cell line can be used for quantitative short-term mutation test and for analyses of mutations at the DNA level. One of these cell lines, AS52, was constructed from a hprt-deficient CHO cell line and transfected with a single functional copy of the E. coli xanthine-guanine phosphoribosyltransferase gene (gpt) stably integrated into a CHO autosome (Tindall et al. 1984). Since the stably expressible gpt gene is integrated into one of the autosomes in CHO cells, the status of the gene is similar to that of the L5187/$TK^{+/-}$ cells and expected to recover more 6-TG^r mutants with deletion or other chromosomal mutations. Results from experiments showed that X-rays induced significantly (6-19 fold) higher mutant frequencies in AS52 cells. Whereas similar 6-TG^r mutant frequencies were observed between the CHO/HPRT mutation assay and the AS52 cell line after treatments with ethyl methanesulfonate (EMS), UV or the frameshift mutagen ICR191. Southern blot hybridization analyses indicated that EMS, UV and ICR 191 induced primarily gene mutations at the gpt and hprt loci, whereas X-rays induced primarily gross deletion mutations at both loci

(Stankowski et al. 1986, Stankowski and Hsie 1986). Recently a similar conclusion was reached from comparative studies of X-ray and EMS induced mutations at hemizygous (+/o) and heterozygous (+/-) aprt loci, in two CHO cell lines, i.e. mutant frequencies were higher in the latter assay than in the former (Bradley et al. 1988). Previously a similar conclusion was reported by Waldren et al. who developed an AL-J1 hybrid CHO cell line containing a human chromosome 11 (Waldren et al. 1979). The extra human chromosome 11 contains three cell surface antigens a1, a2 and a3 that render the cells sensitive to killing by different specific antisera in the presence of complements, providing a selective method for mutants lost these genes. Since none of these genes on the human chromosome 11 is essential for cell survival, mutants due to gene or chromosome mutations, including those with deletions or mutations of other genes on the same chromosome can be viable. The experimental results indicate that X-rays induced mutant frequencies were 200 times more than those obtained at the hprt locus (Waldren et al. 1986).

3. Assays of DNA and chromosomal damages in mammalian cells

Alterations in the structure or number of chromosomes might cause deleterious mutations and result in reproductive cell death or neoplastic transformation. Cytogenetic analyses have revealed a large number of chromosomal aberrations such as translocation, rearrangement, terminal or interstitial deletion, pericentric or paracentric inversion, aneuploidy and

polyploidy in human cancer (reviewed by Klein and Klein 1985, Dalcin and Sandberg 1989). In addition, studies of some human autosomal recessive conditions such as Fanconi's anemia, Bloom's syndrome and ataxia telangiectasia, have revealed that these syndromes are associated with an increased incidence of sister chromatid exchanges (SCE), chromosomal aberrations and neoplasia (reviewed by Arlett and Lehmann 1978, Bohr et al. 1989). For these reasons, chromosomal mutations have been used as endpoints in genotoxic short-term tests for monitoring human exposure to chemical agents with potential carcinogenic activity (IARC 1980). For more than a decade, several genotoxic assays measuring the aneuploidy or DNA damages in mammalian cells have been developed to predict chemical carcinogenicity including chromosome aberration assays (Scott et al. 1983, Galloway et al. 1985), sister chromatid exchange assays (Latt et al. 1981), micronuclei assays (reviewed by Heddle et al. 1983, Choy et al. 1985), and unscheduled DNA synthesis assays (reviewed by Hanawalt et al. 1978).

C. Current uncertainties in carcinogen/mutagen screening

One basic assumption for risk assessment scientists is the familiar phrase included in the preamble of the IARC monographs (IARC 1987): "In the absence of adequate data on humans, it is reasonable for practical purposes, to regard chemicals for which there is "sufficient" evidence of carcinogenicity in animals as if they presented a carcinogenic risk to humans." Clearly, some efforts should be made to

determine whether experimental results in animals are applicable to humans. A set of principles were established for Environmental Protection Agency (EPA) by the National Research Council Committee on Drinking Water and Health to convince the acceptance of animal models by the risk assessment community (NAS 1977 and 1986). These principles for assessment of effects of long-term exposure to carcinogens are the source of uncertainties. These principles are listed in the following: (i) effects in animals, properly qualified, are applicable to humans; (ii) methods do not now exist to establish a threshold for long-term effects of toxic agents; (iii) the exposure of experimental animals to toxic agents in high doses is a necessary and valid method of discovering possible carcinogenic hazards in humans; (iv) agents should be assessed in terms of human risk, rather than as "safe" or "unsafe." The recent results of the National Toxicology Program (NTP) studies of short-term tests have indicated a concern of uncertainty regarding the predictability of short-term tests (Ashby 1986, Clive 1987, Clayson 1987, Tennant et al. 1987 Zeiger 1987). The debate in regards to the uncertainty of rodent bioassays in the last five years has led to much thoughtful discussion about assessment of test results as well as future directions of carcinogens/mutagens screening (Ashby 1988, Ashby and Purchase 1988, Bridges 1988, Brockman and DeMarini 1988, Brusick 1988, Douglas et al. 1988, Ennever and Rosenkraz 1988, Haseman et al. 1988, Heddle 1988, Kier 1988, Ramel 1988, Trosko 1988, Tweats and Gatehouse 1988, Young

1988, Ashby 1989, Ames and Gold 1990b, Cohen and Ellwein 1990, Marx 1990, Zeiger et al. 1990, Weinstein 1991, Ashby and Morrod 1991).

Several lines of evidence have shown that the carcinogenicity differences between test animals and humans created a crucial uncertainty in the assessment of environmental chemicals. For instance, because of the enzymatic difference for chemical toxication/detoxication mechanisms between different species, procarcinogens/ promutagens do not appear to express their activity in every species. An excellent example is isoniazid, which is reproducibly carcinogenic in mice but not in hamsters and probably not in rats. One possible explanation has been suggested that the metabolism of the chemical in mice produces a high level of mutagenic hydrazine but the levels of mutagenic hydrazine are low in other species (Jansen et al. 1980). Similarly, it has been reported that DNA repair mechanisms in rodent cells are different from human cells and these differences are the crucial factors for the process of carcinogenesis (Regan and Setlow 1973). Furthermore, for the convenience of data interpretation, animal bioassays are conducted on highly uniform animals, selected for age, strain, health and population size, which are maintained with a rigidly controlled diet and environment. Consequently, unless more research could be done to understand the difference between experimental animals and humans, we are always confronted with uncertainties.

Another controversy is the feasibility that potential low dose effect to humans can be derived from extrapolation of high dose effect of environmental mutagens on laboratory animals. In a standard rodent lifetime bioassay, the chemicals are usually administered daily to rodents over a lifetime with the Maximum Tolerated Doses (MTDs), which are the highest doses that can be given without causing severe weight loss or other life-threatening signs of toxicity. These doses are much higher, sometimes an order of magnitude more, than the doses to which people are likely to be exposed.

Recent studies by some investigators indicated that the MTD of carcinogens could cause cell death which allows neighboring cells to proliferate and causes oxygen radical production from phagocytosis, thereby resulting in chronic inflammation. Both are important aspects of the carcinogenic process (Bernstein et al. 1985a, 1985b). Ames and Gold (1990b) further indicated that a dividing cell is much more at risk for mutation than a quiescent cell because the fragile single-stranded rather than double-stranded DNA during cell division is more vulnerable to damage which can be converted by DNA repair to stable mutations in a much shorter time during cell division. In addition, cell division may increase the expression of certain oncogenes (Hay 1991).

Rodent lifetime bioassays have identified a seemingly high proportion, 65% of 800 chemicals tested, being labeled as carcinogens (Gold et al. 1984, 1986). Similar experiments with 52 natural pesticides also tested in MTDs, showed that 27 of

these natural chemicals should be considered as rodent carcinogens. These 27 rodent carcinogens have been found in 57 different foods including apples, bananas, carrots, celery, coffee, lettuce, orange juice, peas, potatoes and tomatoes (Ames et al. 1990). It is generally believed that diets rich in fruits and vegetables tend to reduce human cancer. The rodent MTD test that labels many plant chemicals as cancer-causing in humans may be misleading (Abelson 1990).

III. Molecular Analysis of Mutations

Chemical and physical carcinogens induce specific base changes and leave their imprints of damages on DNA. From molecular epidemiology, scientists may find specific DNA damages caused by specific carcinogens in particular types of tumors, and then use the information to identify the precise causative agent. Knowledge of mutation patterns found in human cancer genes allows for predictions to be made based on the likelihood of an exogeneous DNA-damaging agent being involved. Revealing the molecular information from mutated human cancer genes is likely to have a profound impact on our understanding of the origins of human cancer.

A considerable amount of data has indicated that the interaction of ultimate chemical carcinogens and ionizing radiation with specific bases and DNA sequences results in permanent DNA alterations recognized as mutations (Reviewed by Meuth 1990). For example, benzo(a)pyrene exposure often

results in G to T transversion (Mazur and Glickman 1988), melphalan induces predominantly A to T transversion (Wang et al. 1990), MNNG or other alkylating agents modifies the O^6 position of guanine altering its hydrogen-binding properties and produces mainly G to A transitions (Loechler et al. 1984), ICR-191 (a substituted acridine) produces predominantly frameshift mutations (Cariello et al. 1990), and ionizing radiation on the other hand often causes deletions of DNA sequences. Recently, a direct correspondence between the type of mutation seen in a tumor and that expected based on laboratory studies of the putative carcinogen has been reported in humans. Twelve of 13 point mutations of p53 gene reported in hepatocellular carcinoma from patients living in Africa and China where aflatoxins are known risk factors for liver cancer were substitutions of T for G (Bressac et al. 1991, Hsu et al. 1991). Eleven of these p53 mutations found in hepatocellular carcinoma have resulted in an arginine to serine substitution with G to T transversion in codon 249. These observations lead to the speculation that the mutated p53 products might interact with another hepatoma risk factor, hepatitis B or its proteins, and provide a growth advantage in hepatomas (Harris 1991). The importance of these results suggest that G to T transversion by aflatoxin B1 in food contamination is a crucial factor in hepatocarcinogenesis (Foster et al. 1983). Thus the knowledge of the site and nature of mutations in particular tumors might be useful in the determination of the major causative agent of a tumor.

Mutational spectra, the pattern of DNA alterations associated with a mutagen, can provide molecular information as to the environmental causes of human mutations. Southern blotting analysis is useful as a means of assessing the fraction of radiation-induced mutations attributable to major genomic alterations in mammalian cells such as large insertions, deletions or translocations. However, Southern analysis cannot detect deletions or insertions which are less than 50 base pairs (the limitation of resolution of Southern analysis using agarose gel electrophoresis) as well as most base substitution and frameshift mutations (Grosovsky et al. 1988). These concepts have been supported by the induction of mutation in genes essential for cell viability by agents causing base substitutions and in-frame deletions or insertions but not by agents causing deletion mutations. It has been well-documented that the inability of ionizing radiation to induce ouabain resistance, a mutation of the essential Na^+, K^+-ATPase gene, is attributable to the inability to induce point mutations by ionizing radiation in mammalian cells (Arlett et al. 1975, Chang et al. 1978).

The limit of resolution of Southern analysis calls for the direct DNA sequence analysis to understand the molecular mechanisms of mutations. With the help of recombinant DNA technology, investigators have recently developed two basic experimental designs to facilitate target DNA sequence analysis and circumvent the problem of genome complexity. Firstly, the selectable target DNA sequence for the mutagen is

subcloned into an extrachromosomal DNA, a shuttle vector, and introduced into the appropriate host cells. (reviewed by Sarasin 1989a, Vos and Hanawalt 1989) Secondly, the selectable target DNA sequence of an intact endogeneous gene can be analyzed by direct sequencing of PCR-amplified products (Drobetsky et al. 1989).

Shuttle vectors are designed to contain a selectable target gene, which not only can be mutated during replication in cultured mammalian somatic cells but also can be clonally amplified in bacteria for sequence analysis. For the purpose of mutagenesis studies, shuttle vectors contain at least three functional elements: 1) the origin of replication and other information required for replication in mammalian cells, 2) an origin of replication and a drug-resistance marker allowing their replication and maintenance in bacteria, and 3) a marker gene to reveal mutations in bacteria (often by colony colors). Three types of shuttle vector have been developed: 1) a vector contains SV40 origin which can autonomously replicate in the nucleus of transfected cells (Lebkowski et al. 1985); 2) a vector based on Epstein-Barr virus which replicates as a free plasmid in mammalian nuclei but under cellular control (Drinkwater and Klinedinst 1986); and 3) a vector which can integrate into the cellular genome and replicate with chromosomal DNA, but can be specifically isolated from surrounding cellular sequences (Ashman and Davison 1987). It has been reported that SV40-based shuttle vectors can have unexpected spontaneous mutation frequencies as high as 10^{-2}

(Calos et al. 1983, Razzaque et al. 1983). Although this disadvantage can be partly overcome by using a particular cell line as recipient, this has decreased the value of using the shuttle vectors (Lebkowski et al. 1985). Furthermore, the crucial disadvantage of shuttle vector studies lies in the fact that they do not mimic exactly what happens with chromosomal loci in mutagenesis. With the development of polymerase chain reaction techniques for mutagenesis study, shuttle vectors no longer have such a great speed advantage (Sarasin 1989b).

Studies of the alterations produced directly on the cellular genome are particularly important since they reflect mutagenesis occured in the living organisms. Most sequence analysis of such target genes have been centered around four selectable markers, adenine phosphoribosyltransferase (aprt), dihydrofolate reductase (dhfr), hypoxanthine-guanine phosphoribosyltransferase (hprt), and thymidine kinase (tk) (reviewed by Meuth 1990). These genes encode for non-essential or purine/pyrimidine salvage pathway enzymes which allow single step forward selection of drug resistance for enzyme deficient mutants and backward selection of revertants. Two selectable markers, aprt and hprt, are most frequently used target genes for mutagenesis studies involving direct sequencing of PCR-amplified products (Vrieling et al. 1988, Simpson et al. 1988). The small size of the aprt locus (2.0 kb including introns) and the isolation of hemizygous $aprt^{+/0}$ CHO cell lines have simplified the interpretation of experimental

assays such as Southern blotting and sequencing (Lowy et al. 1980, Nalbantoglu et al. 1983). Although the hprt gene is a large selectable marker with the structural gene spread over 44 kb of DNA in human cells (Patel et al. 1986), several reasons have made the hprt gene an even more extensively used selectable marker for mutagenesis studies and sequence analysis. First, the hprt gene resides on the X chromosome, therefore the locus is functionally hemizygous in both male and female cells. Thus the selection of hprt deficient mutants is feasible from virtually any diploid cell line or primary cell without gross chromosomal abnormalities. Second, the in vivo data from studies of a pool of germ line hprt deficient mutations (Lesch-Nyhan syndrome) have advanced our knowledge concerning the biochemical etiology of the syndrome. Therefore molecular mechanisms of mutation in vitro can be compared to that observed in vivo. Finally, hprt has the smallest coding sequence (654 base pairs) among these selectable markers in the 1.6 Kb hprt messenger RNA transcribed from 44 Kb genomic DNA. Using reverse transcriptase, the hprt cDNA can be synthesized from poly(A)$^+$RNA as well as total cytoplasmic RNA, and then cloned into M13 vector for sequencing (Simpson et al. 1988, Vrieling et al. 1988).

For the purpose of studying the spectra of spontaneous or induced mutations in mammalian cells, a simple and rapid method that requires only a few cells is highly desirable to determine the mutational alteration and location for a large number of mutants directly from PCR-amplified products. One

recent report has demonstrated that the hprt cDNA can be detected with ethidium bromide-stained agarose gel electrophoresis from as low as one cell of mutant or normal human fibroblast after reverse transcription of mRNA from cell lysate, followed by two rounds of PCR (30 cycles/round) with two pairs of primers. These double-stranded PCR products can be used directly for sequencing without any subcloning (Yang et al. 1989). Nevertheless, two types of hprt deficient mutants, those with no expression of mutant mRNAs and those with splice junction mutations, are unable to be analyzed by the PCR-amplified cDNA sequencing method. One approach has been used to detect the molecular alterations of splice junction mutant which usually contains a skipped exon of hprt mRNA from the error of RNA splicing (Gibbs et al. 1990). Since the sequence of the entire human hprt gene has been published (Edwards et al. 1990), it is possible to synthesize primers from regions flanking each of the nine exons and examine the hprt gene sequences directly (Gibbs et al. 1990).

MATERIALS AND METHODS

The strategy for developing a sensitive and accurate assay for environmental mutagens

An ideal assay for environmental mutagens should be at once 1) relevant -- able to detect agents mutagenic to human cells; 2) sensitive -- able to detect mutagens at low doses; 3) precise -- able to reveal mutations at the molecular level; and 4) simple and inexpensive. Although these goals may not be attainable in one assay system, assays with several of these attributes should be preferred. With these considerations in mind, I decided to develop a mutation assay using a near diploid human cell line (for goal 1) which has one functional hypoxanthine-guanine phosphoribosyltransferase (hprt) gene on the inactive X-chromosome. Therefore, mutants of hprt gene (6-thioguanine resistant, $6TG^r$) can be recovered at higher frequency after mutagen treatment (goal 2), since the viability of the mutants will not be affected by mutations or deletions of surrounding essential genes on the same chromosome (homologous essential genes are present on the active X-chromosome). These mutants with deleted surrounding essential genes may not be viable if the functional hprt gene is on the active X chromosome, since homologous essential genes are not functional on the inactive X-chromosome. (see

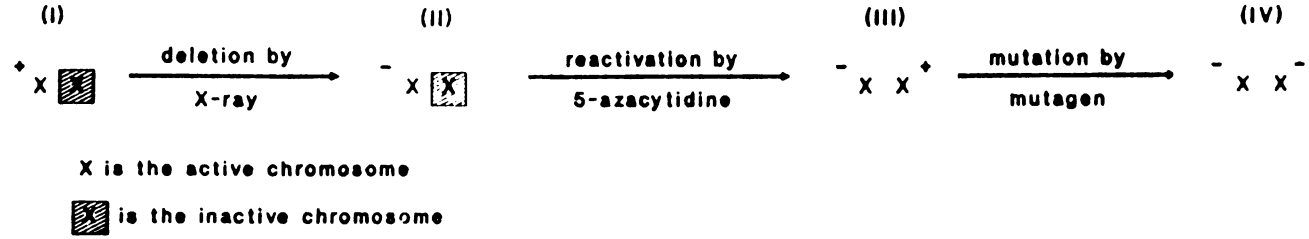
Figure 4). In addition, if the cell line developed can be used to analyze hprt gene mutations at the DNA level, we can precisely understand the specific molecular changes induced by specific agents (goal 3). Since the cell line has high colony-forming efficiency (more than 60%) in a modified Eagle's medium supplemented with low concentration of fetal bovine serum (5%), the quantitative mutation assay and the DNA sequencing of mutant hprt gene using PCR amplified cDNA (if successful) will be simple and inexpensive (goal 4).

Chemicals, medium and culture condition

Human teratocarcinoma-derived cell lines were grown in a modified Eagle's medium (Eagle 1959) (with Earle's balanced salt solution, 50% increase of vitamins and essential amino acids except glutamine ,and 100% increase of non-essential amino acids) supplemented with 1 mM sodium pyruvate and 5% fetal bovine serum (Gibco BRL, Life Technologies, Inc., Gaithersburg MD). The normal human primary fibroblasts were cultured in the same medium but supplemented with 10% fetal bovine serum. The medium was also supplemented with 100 units/ml penicillin G and 100 ug/ml streptomycin (Eli Lilly and Company, Indianapolis, IN) or 50 ug/ml Gentamycin (Quality Biological, Inc., Gaithersburg, MD). All cultures were incubated at 37°C with humidified air in water-jacketed incubators supplied with 5% CO_2. Confluent cells were routinely subcultured in a 1 to 4 ratio using 0.01%

Figure 4. The strategy for developing a sensitive human epithelial cell line for environmental mutagens. The upper diagram shows the mechanisms that more 6-TG^r mutants are recovable in the HTX6TG-1/5AC4-1 cell line [46, XX, t(15; 20) and t(11;X)] than in the parental HTP_3-4 cell line [46, XX, t(15; 20]. The detail explanation is provided in the text. The "+" indicates that the hprt gene is nonfunctional. A, B, and C are genes adjacent to the hprt locus , of them gene B is assumed to be essential for cell survival. Dotted lines indicate the deletion mutations; 6-TG^r and 6-TG^s stand for 6-thioguanine resistant and sensitive, respectively.

The *hprt* Reactivation Strategy



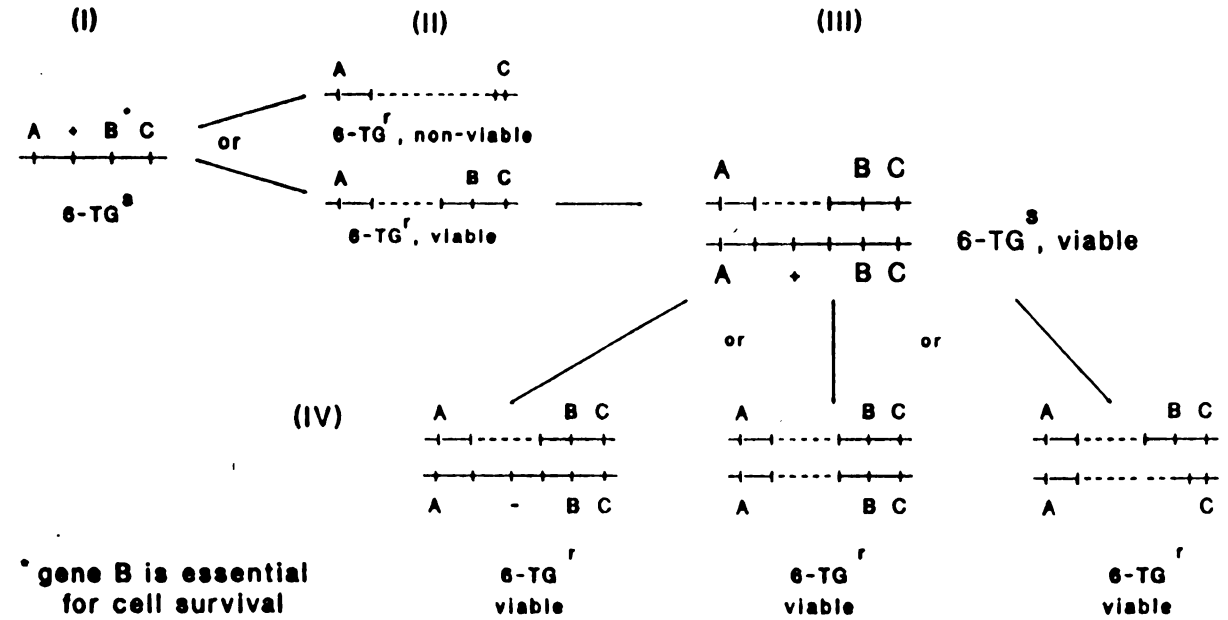


Figure 4

crystalline trypsin in calcium and magnesium free phosphate-buffered saline (PBS) at 37°C for 10 min. For storage of cells, trypsinized cells were centrifuged at 4°C to remove trypsin, resuspended in 10% dimethylsulfoxide (DMSO) in PBS, sealed in glass ampules (Wheaton, Millvilles, NJ) and then frozen in liquid nitrogen until needed for experimentation.

Most chemicals, such as hypoxanthine, amethopterin, thymidine, 2-amino-6-mercaptopurine (6-thioguanine), 5-azacytidine, daunomycin, crystal violet, N-methyl-N-nitro-N-nitrosoguanidine (MNNG) for mutagenesis studies were obtained from Sigma Chemical Co. (St. Louis, MO). Fetal bovine sera were purchased from either Gibco BRL, Life Technologies or Hyclone Laboratories, Inc. (Logan, Utah). Most restriction enzymes or DNA and RNA modifying enzymes were supplied from either Gibco BRL, Boehringer Mannheim Biochemicals (Indianapolis, IN), or New England BioLabs, Inc. (Beverly, MA) unless specifically indicated. Oligonucleotides for hprt polymerase chain reaction and DNA sequencing were synthesized from either National Biosciences Inc (Hamel, MN) or Bio-synthesis, Inc. (Denton, TX). The sequences of hprt PCR and sequencing primers are described as previously reported (Yang et al. 1989), including CTGCTCCGCCACCGGCTTCC (PCR-1), GATAATTTTACTGGCGATGT (PCR-2), CCTGAGCAGTCAGCCCGCGC (PCR-3), and CAATAGGACTCCAGATGTTT (PCR-4) for PCR amplification as well as ATTTCTATTCAGT (seq-1), ATGGGAGGCCATC (seq-2), and TATAATTGACACT (seq-3) for direct nucleotide sequencing.

Plasmid

The p4aA8 plasmid carrying an expressible full-length human hprt cDNA gene was kindly provided by Dr. T. Friedmann of the University of California at San Diego, La Jolla, CA (Jolly et al. 1983).

Cell strains

Three human teratocarcinoma cell lines, HTP_3-4, HTX6TG-1, HTX6TG-1/5AC4-1 were used in this study. The original human ovarian teratocarcinoma-derived cell line (PA-1) was obtained by culturing ascitic fluid cells from a patient with recurrence of malignant ovarian teratoma (Zeuthen et al. 1980). A clone derived from PA-1 designated P3 exhibits an epithelial cell morphology, a pseudodiploid karyotype [46, xx, t(15;20)] and high colony forming efficiency, is a gift from Dr. E. Huberman (Argonne National Laboratory). A subclone of P3 with higher colony forming efficiency (65%) and tight uniform colonies, designated HPT_3-4, was isolated from a single colony by the glass cylinder method (Ham and Puck 1962). The normal human primary fibroblast cultures (NF79-2) used in this dissertation were established by this laboratory from newborn foreskins.

Selection of a non-revertible 6-TG^r mutant which may contain a deleted hprt gene on the active X-chromosome

As shown in Figure 4, HTP_3-4 presumably contains two X chromosomes with only one functional X chromosome expressing

the de novo purine synthesis salvage enzyme hprt, and is able to survive in HAT medium ($1x10^{-4}$ M hypoxanthine, $2x10^{-6}$M amethopterin and $2x10^{-5}$M thymidine) (i.e., hprt$^+$). As a first step toward developing a cell line with higher sensitivity for mutation assay, the HTP_3-4 cells were treated with X-rays using a Torrex 150-kV cabinet X-ray machine to delete the hprt gene on the active X-chromosome and selected for 6-TG^r mutants in 6-thioguanine (10ug/ml) containing medium (Kavanagh et al. 1986). One clone with 6-thioguanine resistance phenotype, designated HTX6TG-1, was tested for spontaneous reversion frequency in order to confirm that it might contain a deletion mutation of the hprt gene. A total of $5x10^6$ HTX6TG-1 cells were plated in 24 dishes (9 cm) in medium containing HAT to detect spontaneous reversion frequency of HTX6TG-1 to HAT^r cells. The HTX6TG-1/5AC4-1 cells were derived from non-revertible 6-TG^r HTX6TG-1 cells by the treatment of 5-azacytidine to reactivate the methylated hprt gene on the inactivated X-chromosome and by selection in the HAT medium (hprt$^+$). In contrast to the parental HTP_3-4 cell line or normal human cell lines, the reactivated hprt gene is on a non-essential X-chromosome. Therefore, deletions or mutations of other genes on the non-essential chromosome associated with the hprt gene mutation would not affect the survival of a 6-TG^r mutant. After exposure to mutagens, a higher frequency of induced 6-TG^r mutants might be expected.

The hprt gene reactivation by 5-azacytidine treatment

The nonrevertible 6-TG^r HTX6TG-1 cells were seeded at $2.5x10^5$ cells per dish (9 cm) for overnight. Cells were treated with freshly prepared 5-azacytidine (2 uM) for 24 hours. Cultures were washed with PBS to remove 5-azacytidine, changed with new medium and incubated for 3 days to allow for phenotypic expression. HAT^r (hprt$^+$) colonies were selected and developed in the HAT medium for two weeks (Harris 1982, 1984). Fifteen colonies (designated HTX6TG-1/5AC) were isolated by the glass cylinder method and incubated in the HAT medium. For determination of reversion frequencies of hprt genes at the reactivated HTX6TG-1/5AC clones, each clone was plated with $2x10^5$ cells per dish (9 cm) and incubated in 6-TG containing medium for two weeks. The lower the number of 6-TG^r colonies indicates the more stable of the reactivated hprt gene of a clone is indicated and the more suitable the clone can be used for mutagenesis studies. One clone, designated HTX6TG-1/5AC4, with relatively low reversion frequency from HAT^r to 6-TG^r was chosen for further studies. Since the frequency of spontaneous reversion from HAT^r to 6-TG^r for the HTX6TG-1/5AC4 cell line was not ideal (about $1x10^{-4}$), the subcloning of the cell line was undertaken to select for a low spontaneous reversion clone. The HTX6TG-1/5AC4-1 subclone of HTX6TG-1/5AC4 was found to have low spontaneous reversion frequency (less than $5x10^{-5}$) and was chosen for further mutagenesis studies.

Mutation induction at the hprt locus

In order to determine whether the HTX6TG-1/5AC4-1 cell line has enhanced mutant frequency for other mutagenic treatment, the two HAT^r (i.e. $hprt^+$) cell lines, HTP_3-4 and HTXGTG-1/5AC4-1, were treated with same mutagens under same conditions and compared for frequencies of induced mutations. Three mutagens (X-ray, ultraviolet light, MNNG) and one clastogen (daunomycin), each induced mutation through different mechanisms, were used in the quantitative mutation studies. The treatments for each of these agents are described as follows. 1) X-ray induced mutation: the two $hprt^+$ cell lines cultured in actively growing stage, were trypsinized, cell number counted and suspended in medium. Except during the time of irradiation, cells were kept on ice after trypsinization until time for cell plating. Cells were irradiated in suspension with various doses of X-rays (0, 100, 300 and 500 rads) generated by a Torrex 150-KV cabinet X-ray machine operated at 150 KV and 5 mA without a filter. 2) Ultraviolet light (UV) induced mutation: the two $hprt^+$ cell lines were trypsinized and replated in 9 cm plates for attachment for overnight before UV-irradiation. With the medium removed from the plates, the attached cells were exposed to various UV doses (0, 1.5, 4.5 and 7.5 J/m^2) from a germicidal lamp (G25 T8-25W) which was positioned to deliver a dose rate of 10 to 15 $erg/mm^2/sec$ (1 to 1.5 $J/m^2/sec$). 3) MNNG-induced mutation: the two $hprt^+$ cell lines were

trypsinized and replated in 9 cm plates for more than 4 hours for attachment, and then incubated with various concentrations (0, 2.5, 5 and 10 mM) of N-methyl-N'-nitro-N-nitrosoguanine (MNNG) for 4 hours in growth medium. 4) Daunomycin-induced mutation: the two hprt$^+$ cell lines were trypsinized and replated in 9 cm plates for overnight and incubated with various concentrations (0, 25, 50 and 75 ng/ml) of daunomycin for 3 hours.

Selection and quantitation of 6-thioguanine resistant (6-TG^r) mutants

After the removal of mutagens, cells were replated for determinations of dose-responsive survival and for mutation expression. Small portions of the cells were serially diluted to desired cell densities; 1000 cells were plated in three 9 cm plates with normal growth medium for the determination of cell survival. The majority of the cells were replated in 75 cm^2 flasks and incubated in growth medium for mutation expression. With routine medium changes and subcultures once every two days, the cells were allowed to grow for 8 days and 12 days after mutagen treatment for mutation expression before the cells were replated for quantitative mutation assays (O'Neill and Hsie 1977, O'Neil et al. 1977). In a typical mutation assay for 6-TG^r mutants, cells were trypsinized and replated in three 9 cm plates at low cell density (1,000 cells/three plates) for colony forming efficiency assay and in ten 9 cm plates at higher cell density (1 x 10^5 cells per

plate) for assay of 6-TG^r mutants. Cells plated for mutation assay were incubated in medium containing 6-thioguanine (10 ug/ml) for two weeks with 6-TG medium change once four days after cell replating. In X-ray-induced mutation studies, normal human fibroblasts were also included as normal cell control. Since primary fibroblasts have a larger cell size and slower growth rate, lower cell density (200 cells per plate) was used to determine colony-forming efficiency and $5x10^4$ cells per plate were used for 6-TG^r mutants assay to avoid metabolic cooperation which may result in 6-TG^r cell death when toxic 6-TG metabolites are transferred from 6-TG sensitive (6-TG^s) cells through gap junctions (Cox et al. 1970). The 6-TG^r mutants from normal human fibroblasts were allowed to grow for 3 weeks with medium change every 7 days. The surviving colonies in both survival and mutation assays were stained with crystal violet (1% in 10% ethanol solution) and quantitated. The mutant frequency is calculated by dividing the total number of 6-TG^r mutants recovered in each treatment by the total number of cells plated corrected by the colony-forming efficiency determined for these cells.

Extraction of genomic DNA

The isolation of high molecular weight DNA from various human teratocarcinoma cell lines was according to the standard method (Maniatis et al. 1982) with some modifications. Cells from 150 cm^2 cultured flasks were washed with PBS, trypsinized

and centrifuged at 1,000 rpm for 10 minutes. The cell pellets were rinsed and centrifuged again to remove all trypsin and serum proteins. Cell pellets could be stored in -70°C freezer for several months or processed right away by suspending in 4.25 ml T.E. (10mM Tris, 1mM EDTA, pH 8.0) thoroughly in a 50 ml centrifuge tube (Corning Laboratory Sciences Co., Wexford PA). The resuspended cells were incubated with 50 mM EDTA, 0.5% sodium dodecyl sulfate (SDS) and 250 ug/ml proteinase K (Boehinger Mannheim Biochemicasl, Indianapolis, IN) in 5 ml total volume and incubated at 50°C for overnight with gentle shaking. Proteinase K-digested cell lysate was gently extracted twice with an equal volume of phenol (saturated with T.E.)/chloroform mixed with isoamyl alcohol 24 : 1 and once with chloroform then centrifuged at 10,000 rpm for 5 min for phases separation. The aqueous phase containing genomic DNA was collected in dialysis tubing for overnight dialysis against 4 liters of T.E.. DNA samples were then treated with 100 ug/ml of DNase-free RNase (Sigma Chemical Co., St. Louis, MO) at 37°C for one hour. Additional proteinase K digestion was usually proceeded to remove remaining protein contamination in DNA samples again. The phenol/chloroform extraction and dialysis were employed to remove small protein residues and SDS from cellular genomic DNA.

Southern blotting and hybridization

Fifteen ug genomic DNA isolated from human teratocarcinoma cell lines and 6-TG^r mutant clones were cleaved with restriction enzyme (5 units per ug genomic DNA) at 37°C for 5 to 12 hrs. After precipitation with ethanol, restriction enzyme-digested genomic DNA was redissolved in loading buffer (2.5% Ficoll 400, 0.025% bromophenol blue and 0.025% xylene cyanol), applied into slots of 0.7% agarose gel, and run at 32 volts for 10 hours in 1X TBE buffer (0.09M tris-borate and 0.002 M EDTA). The gel was stained in 0.5 ug/ml ethidium bromide and photographed with 667 or 665 Polaroid films. Before denaturation of genomic DNA, the gel was soaked in 0.25 N HCl for 10 minutes twice in order to improve the transfer of DNA fragments which are larger than 15 Kb. Denaturation of genomic DNA was done by soaking the gel for 45 minutes in 0.5 N NaOH and 1.5M NaCl with gentle agitation and solution change once. Subsequently, the gel was neutralized in 1M Tris (pH 8.0) and 1.5M NaCl for 45 minutes with gentle agitation and with one solution change. After neutralization, standard Southern blotting was set up for capillary transfer of denatured genomic DNA to nylon membrane Hybond-N, (Amersham Corporation, Arlington Heights, IL) with 10X SSC (1.5M NaCl and 0.15M Na_3 citrate. $2H_20$) for 24 hours (Southern 1975). Genomic DNA-bound nylon membrane was rinsed with 5X SSC briefly, and then allowed to dry in room temperature for 30 minutes. UV-crosslinking for 4.5 minutes on a UV

Transilluminator (Ultra-violet products, Inc., San Gabnel, CA) was used to immobilize genomic DNA on nylon membrane instead of vacuum baking at 80°C for 2 hr. Nylon membranes were then sealed in hybridization bag either for storage at room temperature or for hybridization with probe immediately.

DNA probe was labeled with [α-^{32}p]dCTP (3000 Ci/mmole, Dupont-NEN research products, Boston, MA) using random primer labeling reaction (Feinberg and Vogelstein 1983, 1984). One hundred ng of DNA was denatured by heating to 95-100°C for 5 minutes in reaction buffer containing 90 mM HEPES (pH 6.6), 2mM Dithiothreitol (DTT), 10mM $MgCl_2$ and 37.5 ng 6-bp random primer (New England Biolabs, Inc., Beverly, MA). This mixture was chilled on ice for 3 minutes, added with 0.4 mM each of dTTP, dATP, dGTP and 100 mCi of [α-^{32}p]dCTP, and then 5 units of Klenow fragment of DNA polymerase I (Bethesda Research Labs, Gaithersburg, MD). The random primer labeling reaction was incubated at 37°C for 30 minutes. Labeled DNA was purified by bio-spin 30 chromatography column (Bio-Rad Laboratories, Richmond, CA) to remove unincorporated [α-^{32}p]dCTP.

A typical prehybridization reaction with 12 x 15 cm^2 surface area of nylon membrane was carried out in 10 ml of solution containing 5X Denhardt solution (0.2% each of Ficoll 400, polyvinylpyrolidone and Fraction V of bovine serum albumin), 6X SSPE (1.07M NaCl, 0.06M sodium phosphate and 0.006M EDTA, pH 7.4), 0.5% SDS, 0.3 mg/ml sheared and denatured salmon sperm DNA, 10% Dextran sulfate, and then

incubated at 65°C for at least 2 hr. Following the addition of heat-denatured labeled DNA probe, hybridization was carried out overnight at 65°C. The membranes were washed with 2X SSC at room temperature twice for 5 minutes each, 1% SDS in 2X SSC twice at 65°C for 30 minutes each, and then 0.05% SDS in 0.1X SSC at room temperature (two washes of 30 minutes each). Autoradiography was carried out at -75°C using Kodak X-omat AR-film and cassette with regular intensifying screens. For rehybridization of the same membrane with different labeled probes, the nylon membrane was not allowed to dry in the wash and autoradiography procedures. The removal of probe was carried out in 50% formamide, 0.1% SDS and 2X SSC at 75°C for one hr with strip solution changed once. After several washes of 2X SSC and confirmation of probe removal with radioactive monitor, the nylon membrane is ready for prehybridization and hybridization again.

Complementary DNA synthesis directly from 6-TG^r mutant lysate

Several precautions are required for successful cDNA synthesis from cell lysate. First, sterile, RNase-free and disposable plasticware is essential for cDNA synthesis. In addition, general laboratory glasswares are often a source of RNase contamination and was baked at 250°C for 4 or more hrs. Finally, all solutions including double-distilled water, $MgCl_2$, PBS should be treated with 0.1% diethylpyrocarbonate (DEPC) for 12 hr at 37°C and autoclaved in liquid cycle for 15

minutes. Gloves was worn at all stages during the experiment and all RNase-free regents, glasswares as well as plasticwares were set aside and stored in a designated cabinet.

The 6-TG^r mutant cells from either cell monolayer or a single colony can be used for cell lysate preparation and cDNA synthesis. Cells were rinsed with PBS, trypsinized, and counted for cell number with hemacytometer. After serial dilution of cells with cold and RNase-free PBS, the desired cell number (usually 500 cells) was pipetted into a 0.5 ml microcentrifuge tube with a total volume of 0.5 ml in RNase-free PBS. Cells were centrifuged at 4°C for 10 minutes and the supernatant was aspirated carefully and completely with a micropipette tip. The invisible cell pellet was added with 5 ul reverse transcription cocktail containing 1X reaction buffer of Moloney murine leukemia virus (M-MLV) reserve transcriptase (50 mM Tris-HCl pH 8.3, 75 mM KCl and 3mM $MgCl_2$), 10 mM DTT, 0.5 mM each of dGTP, dATP, dTTP and dCTP, 10 ng/ul oligo$(dT)_{12-18}$ (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) 100 ng/ul DNase and RNase free bovine serum albumin (Pharmacia LKB Biotechnology Inc.), 1 unit/ul Ribonuclease inhibitor (Promega Biotec, Madison, WI), 2.5% NP-40 (BDH Biochemical Reagents Limited, Poole, UK) and 20 units/ul M-MLV reverse transcriptase(Gibco BRL, Life Science Technologies, Inc.). The invisible cell pellet and 5 ul reverse transcription cocktail were mixed with a micropipette thoroughly to keep it bubble-free and then incubated at 37°C

for at least 1 hr (up to 3 hr). The cDNA synthesis should have a negative control with 5 ul reverse transcription cocktail without cells after the removal of 0.5 ml RNase-free PBS in a microcentrifuge tube. The reactions were stopped on ice and the synthesized cDNA was subsequently subjected to polymerase chain reaction.

The hprt cDNA amplification by using polymerase chain reaction (PCR)

In order to determine the mutation on the hprt coding sequence, two rounds of polymerase chain reactions (each round containing 30 cycles of PCR) using two pairs of primers derived from terminal sequences on both sides of a section of the hprt gene is required to amplify enough quantity of hprt cDNA. The sequences of primers 1 and 3 were selected from coding strand based on the cDNA sequence published by Jolly et al. (1983). The sequences of primers 2 and 4 are from the complementary strand to the hprt coding sequence. The pair of primers 1 and 2 were used to amplify the hprt cDNA in the first round of PCR and another pair of primers 3 and 4 complemented to the first-round PCR products were used to synthesize more hprt cDNA for sequence determination.

The reaction mixtures from negative control and the synthesized single-stranded cDNA were added with PCR reaction cocktail to a total volume of 50 ul. The PCR reaction mixtures were overlaid with 50 ul paraffin oil and performed the amplification in a DNA Thermocycler (Perkin Elmer Cetus

Norwalk, CT) with denaturation in 94°C for 5 minutes (time-delay file) and 30 cycles of polymerase chain reactions with denaturation at 96°C for 1 minute, annealing reaction at 50°C for 1 minute, and polymerization at 72°C for 2 minutes (step-cycle file). After 30 cycles of PCR, the reactions were continued with another polymerization at 72°C for 7 minutes (time-delay file) and finally maintained at 4°C to protect PCR products (soak file). These files were edited to connect all reactions in order and programmed to perform the amplification automatically. The PCR reaction cocktail contains 1X Taq polymerase reaction buffer (150 mM Tris-HCl pH 8.6, 600 mM KCl and 27.5 mM $MgCl_2$), 0.4 mM each of dATP, dGTP, dTTP and dCTP, 0.15 uM (1 ng/ul) of PCR primers, and 2.5 units of Taq polymerase (Perkin Elmer Cetus). After first round of PCR amplification, 5 ul (10%) of each sample were delivered to another new set of 0.5 ml microcentrifuge tubes for second round of PCR in exactly the same condition as the first round of PCR except the addition of PCR primers 3 and 4 instead of 1 and 2. It is necessary to include a separate positive control reaction by using 1 pg of hprt cDNA to assess the performance of amplification. A portion (10%) of the PCR products was examined by electrophoresis of ethidium bromide-stained agarose gel (1.5%) to estimate the efficiency of the PCR amplification and to quantitate roughly the concentration of amplified hprt cDNA.

Chain termination sequencing of double-stranded PCR products

Since the resolution of sequencing gel with two loading of samples is about 230 base pairs, three sequencing primers are necessary to determine the 650 nucleotides of the hprt cDNA sequence.

For obtaining highly specific 5'-end labeling of sequencing primers, the [γ-^{32}p]ATP with highest specific activity (6000 Ci/mmole, Dupon-New England Nuclear, Boston, MA) is required. The kination reaction was carried out in 1X kinase buffer (50 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$ and 15 mM DTT), 7.5 ng/ul sequencing primer, 2.5 uCi/ul [γ-^{32}p]ATP and 0.5 unit/ul T4 DNA polynucleotide kinase at 37°C for 30 minutes. The unincorporated [γ-^{32}p]ATP was eliminated by passing through Nu-Clean D25 disposable spun column (International Biotechnologies Inc., New Haven, CT). It is important to obtain labeled sequencing primers with high specific activity of more than 1.5 x 10^6 cpm per pmole in order to obtain the clear banding of nucleotide sequencing after autoradiography.

Before the sequencing reaction of hprt cDNA, the amplified double-stranded DNA was further purified with microconcentrator Centricon™-30 (Amicon Corp. Beverly, MA) which removed the remaining PCR primers and impurity by centrifugation at 4°C twice with 0.1X T.E. as dilution solution. The hprt cDNA templates (approximately 100 ng) were mixed with labeled sequencing primer (about 2-5x10^6 cpm/2

pmole) in 1X sequencing buffer (100 mM Tris-HCl pH 7.5, 50 mM $MgCl_2$ and 75 mM DTT), denatured by heating to 95°C for 5 minutes, and then rapidly quenching on ice for annealing. The template/primer mixture (14 ul) were added with 3 units of diluted Sequenase version II (1 ul) (United States Biochemical Co., Cleveland, OH) and equally divided into four tubes which were labeled "G","A","T" or "C". To each of these tubes, 2.5 ul of the appropriate dideoxy/deoxy-nucleotides mixture were added. The concentration of dideoxy/deoxy-nucleotides mixture in each designated tubes were described as follows: tube G is 150 mM of each deoxynucleotides (dATP, dGTP, dTTP, dCTP) and 7.5 mM dideoxy GTP; tube A is 150 mM of each deoxynucleotides and 5 mM dideoxy ATP; tube T is 150 mM of each deoxynucleotides and 5 mM dideoxy TTP; tube C is 150 mM of each deoxynucleotides and 7.5 mM dideoxy CTP. The reaction mixture were gently mixed, spun briefly and incubated at 37°C for 15 minutes. Finally, all reactions were quenched on ice and stopped with 4 ul of loading buffer containing 93.3% deionized fomamide, 0.15% xylene cyanol, 0.15% bromophenol blue, and 0.37% EDTA pH 8.0. Since the highly specific labeling primers were used in sequencing reaction, samples can only be stored at -20°C for less than 48 hours prior to loading on a gel without significant degradation.

Gel electrophoresis of DNA sequencing

Glass plates, spacers, combs and the sequencing apparatus with aluminum plate used in the experiments were purchased from International Biotechnologies Inc. (New Haven, CT). Before the shorter glass plate was siliconized with 5% dichlorodimethylsilane in chloroform in a chemical hood, both glass plates were meticulously cleaned using a soapy sponge, rinsed thoroughly with distilled water then with ethanol and carefully wiped dry with Kimwipes to remove any residual particles. The siliconized plate is usually good for at least 6 sequencing gels and able to prevent the gel from sticking to both glass plates, which might have high possibility to tear apart the gel after electrophoresis is completed. Before every gel sandwich was formed, both glass plates were meticulously cleaned first with distilled water, then with ethanol, and wiped dry with Kimwipes to make sure no dust, grease or residual particles are present. The gel sandwich was formed using two glass plates, two 0.4 mm thick side spacers, and one 0.4 mm bottom spaces. To prevent the leakage of acrylamide solution from the assembled gel mold, four corners of glass plates with the connection edges of spacers were sealed with a small amount of silicon grease then several large bulldog binder clips were used to clamp together glass plates on both sides and bottom.

Six percent sequencing gels were prepared as follows: a 40% stock solution of acrylamide/bisacrylamide (38:2) (Bio-Rad

Laboratories, Richmond, CA) was prepared, filtrated with Whatman No. 1 filter paper (Whatman Laboratory Products Inc., Clifton, NJ), and stored in a brown bottle at 4°C. For a 6% sequencing gel with a total volume of 70 ml, 29.4g ultrapure electrophoresis grade urea (Boehringer-Mannheim Biochemicals, Indianapolis, IN), 7 ml 10X TBE, 10.5 ml 40% acrylamide/bisacrylamide solution and 30.5 ml distilled water were mixed and stirred to dissolve solids. To the acrylamide/urea solution, 500 ul of ammonium persulfate and 50 ul TEMED (N,N,N',N'-tetramethylethylenediamine) were added. The mixture was then poured into gel sandwich between glass plates. The gel was carefully examined for air bubbles. The cast of gel usually took about 30 minutes. Acrylamide gels were usually pre-run for overnight with constant 250 volts or 30 minutes to one hour with constant 70 watts. Before samples were subjected to electrophoresis with constant 70 watts, the wells were cleaned with running buffer (1X TBE) in syringe to remove urea. Before loading, the samples were heated at 95°C for about 5 minutes for denaturation. The second loading of samples was done when the slow moving dye (xylene cyanol) was traveled 2/3 of the total gel length. When electrophoresis was run for 4 hr and bromophenol blue was just run out, the gel was separated with glass plates, dried in gel dryers (Bio-Red Laboratories, Richmond CA) at 80°C for 2 hr in vacuum, and exposed to Kodak XAR-5 X-ray film in cassette using two regular intensifier screens at -70°C.

Chromosome analysis of human teratocarcinoma cell lines

Karyotyping and chromosome analysis were carried out by Ms. Sirinunt Hope of the Michigan State University Cytogenetics laboratory, under the direction of Dr. J.V. Higgins. Cells were trypsinized and dropped on a sterile cover glass inside a petri dish for spreading of chromosomes after fixation. After cultured in growth medium overnight, cells were incubated with 0.5 ug/ml colcemid at 37°C for 30 minutes to accumulate cells at metaphase. The treatment of hypotonic solution (0.7% sodium citrate), the fixation of chromosomes with freshly prepared Carnoy's Fixative (3:1 of methanol:acetic acid), the chromosome banding by Leishman's stain for 1.5 minutes, and chromosome analysis with a computer program were carried out by the TECAN machine which is an automatic chromosome analysis apparatus. Some selected metaphases were photographed and developed for precise karyotyping.

RESULTS

The determination of non-revertibility of the 6-TG^r mutant cell line, HTX6TG-1

The genetic determination of non-revertibility of the 6-TG^r mutant HTX6TG-1 cell line is necessary to confirm that it contains a deletion mutation at the hprt gene. There is good reason to believe that the HTX6TG-1 cell line contains a deletion at the hprt gene, since the 6-TG^r mutant was induced by X-rays which have been shown to induce primarily deletion mutations. The results from an experiment to determine the spontaneous frequency of the HTX6TG-1 cell line showed that no HAT^r colony was recovered from 4.8×10^6 cells tested (the spontaneous reversion frequency is less than 2×10^{-7}). Therefore, the mutant hprt gene on the active X chromosome of the HTX6TG-1 cell line appears non-revertible to wild type. This conclusion is supported by the molecular analysis of X-ray induced 6-TG^r mutants in Figure 16 which indicates that the HTX6TG-1 cell line contains a deletion from exon 7 to 9 of the hprt gene (see results presented later).

Reactivation of a hypermethylated hprt gene in the HTX6TG-1 cell line with the treatment of 5-azacytidine

For the purpose of creating a functional hprt gene on the inactive X-chromosome in a cell line with deleted hprt gene on the active X-chromosome, experiments were carried out to reactivate the hypermethylated hprt gene on the inactive X-chromosome of the HTX6TG-1 cell line with the treatment of 2 uM 5-azacytidine for 24 hours and to select for surviving colonies in HAT-containing medium (Harries 1982 and 1984, Turker et al. 1984). The results indicate that the treatment reduced cell survival to 42%, and induced cells with HAT^r phenotype at the frequency of 18.2×10^{-6} (a total of 60 HAT^r clones found in 3.3×10^6 viable cells tested). Compared to the HTX6TG-1 cell line the 5-azacytidine increased the induction of HAT^r phenotype by more than 142 times. However, most of HAT^r clones induced by 5-azacytidine were unstable as evidenced by high reversion frequencies observed for these clones. In order to select for clones with low reversion frequencies, fifteen HAT^r clones (HTX6TG-1/5AC) isolated from the experiment with 5-azacytidine treatment were replated in 6-TG containing medium to determine the spontaneous reversion frequencies for these reactivated hprt genes. The experimental results show that most HAT^r clones tested have high spontaneous reversion frequencies (confluent cells in 9 cm plates were found after 8 days culture from 2×10^5 cells) except the HTX6TG-1/5AC2 and HTX6TG-1/5AC4 clones (less than 8.3×10^{-7}). Further subcloning of HTX6TG-1/5AC4 cells was performed to select for stable

clones with low spontaneous reversion frequencies. The subcloning resulted in the isolation of HTX6TG-1/5AC4-1 which had a reversion frequency lower than $5X10^{-5}$. Karyotype analysis of HTX6TG-1/5AC4-1 cells revealed that the low spontaneous reversion frequency might be attributed to additional translocation between 11 and X chromosomes (see results in the following section).

Karyotypes analyses of human teratocarcinoma-derived cell lines

Karyotype analysis of the HTP_3-4 cell line (Figure 5) showed that the parental cell line is near diploid with one reciprocal translocation (46, XX, t(15;20)] similar to the original cell line, PA-1 reported before (Zeuthen et al. 1980, Huberman et al. 1984). Furthermore, despite the X-ray irradiation and 6-TG^r phenotype, the karyotype of HTX6TG-1, especially X chromosomes, remains [46, XX, t(15;20)] (Figure 6) indistinguishable from the parental HTP_3-4 cells. Since a near normal karyotype of a human epithelial cell line is desired for a human cell mutation assay, the karyotype of the HTX6TG-1/5AC4-1 cell line was also analyzed to confirm that it was derived from the HTP_3-4 cell line and remains near normal in karyotype. The presence of marker chromosomes, reciprocal translocations of chromosome 15 and 20, in HTX6TG-1/5AC4-1 cells clearly confirms that it was derived from the parental HTP_3-4 cell line. However, an additional translocation between chromosome 11 and X was found in the HTX6TG-1/5AC4-1 cell

Figure 5. The karyotype of the HTP_3-4 cell line [46,XX,t(15; 20)].

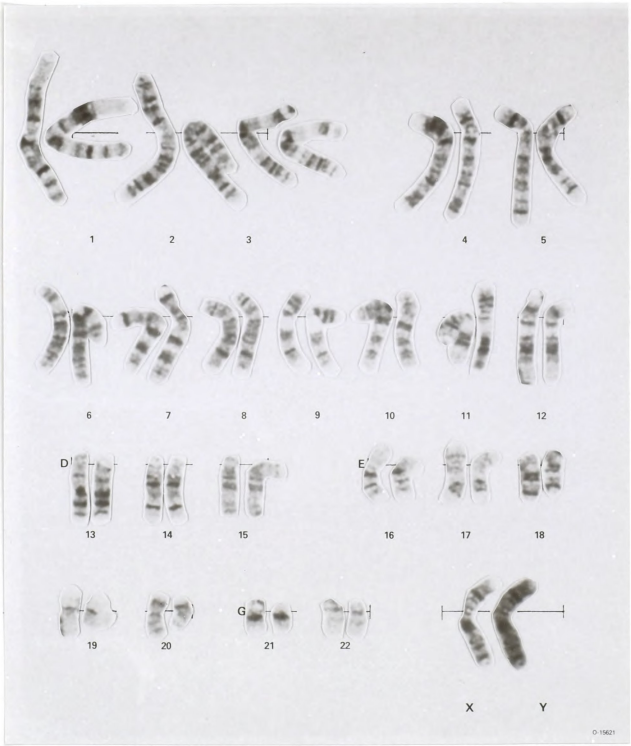


Figure 5

Figure 6. The karyotype of the HTX6TG-1 cell line [46,XX,t(15;20)].

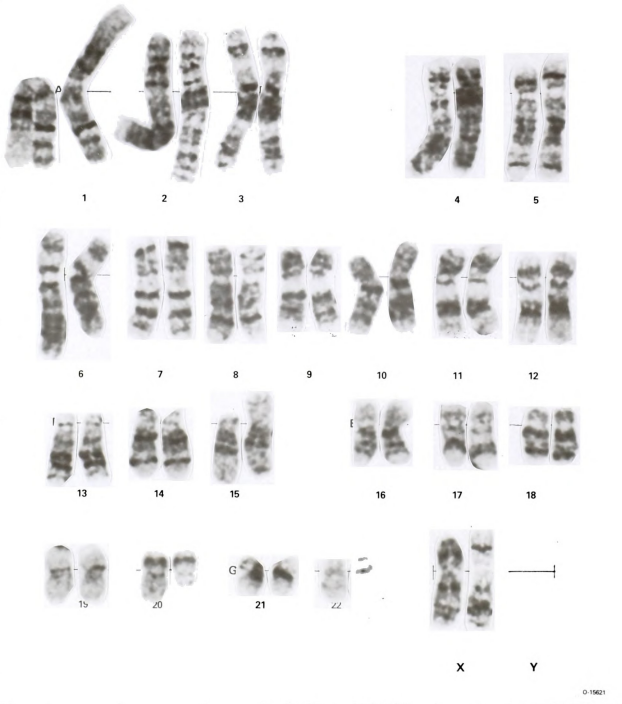


Figure 6

line, otherwise the karyotype is near normal [46, XX, t(15;20) and t(11;X)] (Figure 7). An examination of the banding patterns of the t(11;X) chromosomes indicates that the breakpoint is near Xq27 where the hprt gene is located.

Comparison of X-ray induced mutant frequencies between HTP_3-4 and HTX6TG-1/5AC4-1

To determine whether the HTX6TG-1/5AC4-1 cell line has higher induced mutation frequencies than that of the parental HTP_3-4 cell line, the well known physical ionizing radiation mutagen, X-rays, was first employed to treat both HAT^r cell types to induce 6-TG^r mutants. The results from two separate experiments on cell survival (Figure 8) and mutation inductions (Figure 9) after treatment with various doses of X-rays were presented. As shown, the dose response curves of cell survival between HTP_3-4 and HTX6TG-1/5AC4-1 cell lines appear similar and comparable. The normal primary human fibroblast (NF79-2) appears to be slightly sensitive to X-rays compared with the two HAT^r human teratocarcinoma cell lines, this difference may not be real since both cell lines have previously been found to have normal and comparable X-ray sensitivity to other primary human fibroblasts established in this laboratory. Low mutant frequencies were induced by X-rays in the HTP_3-4 cell line and normal human fibroblasts. In contrast, the mutant frequencies induced by various X-ray doses (0-500 rods) in the HTX6TG-1/5AC4-1 cell line were strikingly increased to as high as 50 fold (500 Rads) more

Figure 7. The karyotype of the HTX6TG-1/5AC4-1 cell line [46,XX,t(15; 20) and t(11;X)].

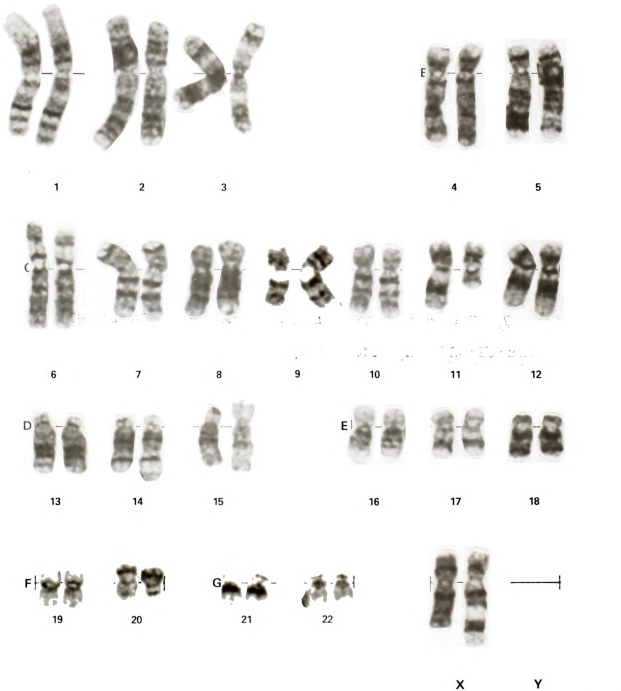


Figure 7

Figure 8. X-ray survival curves for two HAT^r-human terotocarcinoma cell lines. Solid circles and triangles represent two separate experiments from the HTX6TG-1/5AC4-1 cell line; Open circles and triangles represent two separate experiments from parental HTP_3-4 cell line; and open squares stand for results obtained from the normal human fibroblast strain, NF79-2.

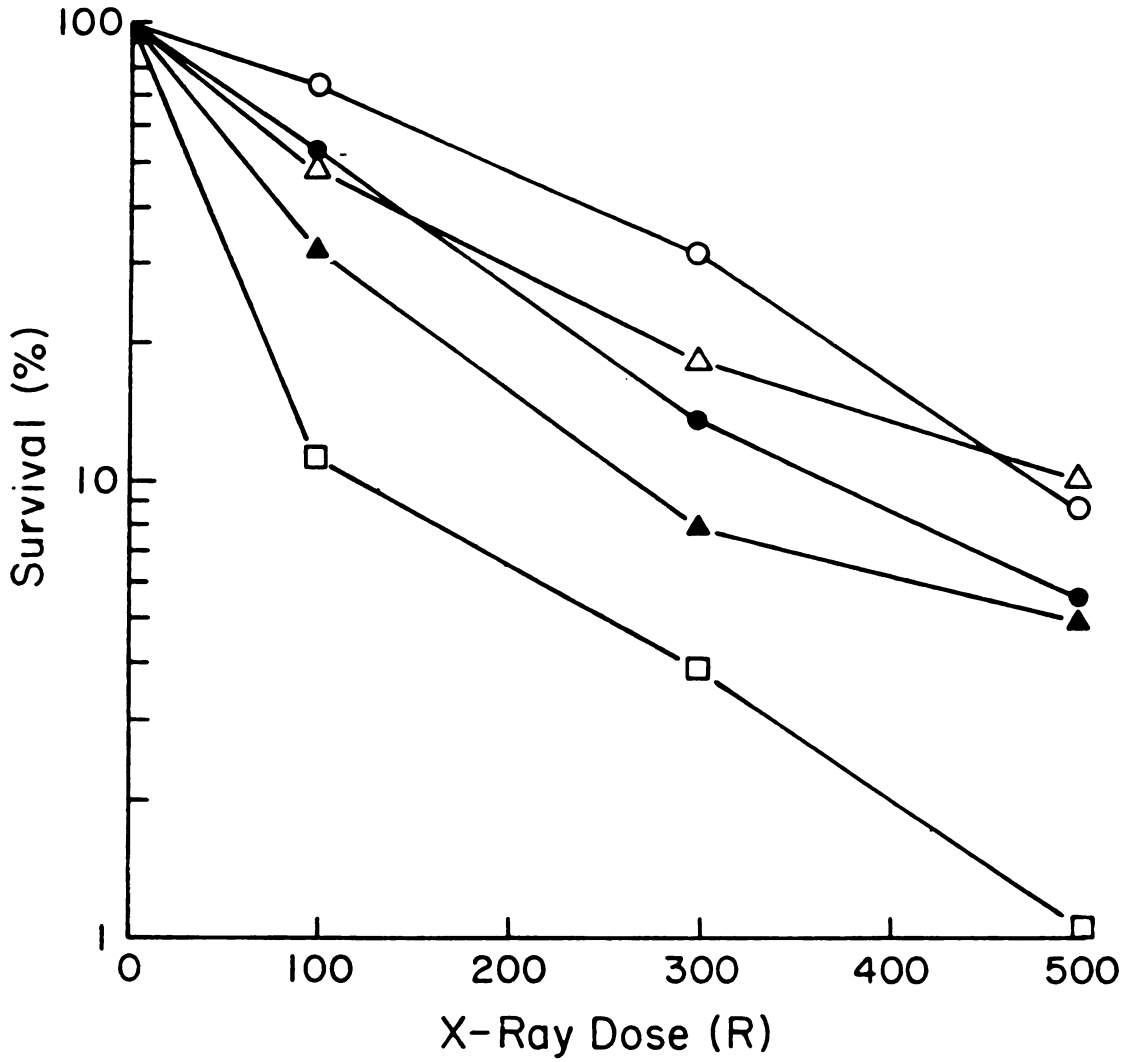


Figure 8

Figure 9. Mutation induction curves by X-rays for two HAT^r-human teratocarcinoma cell lines. Solid circles and triangles represent two different experiments from the HTX6TG-1/5AC4-1 cell line; Open circles and triangles represent two different experiments from the parental HTP_3-4 cell line; and open squares stand for an experiment using the normal human fibroblast strain, NF79-2.

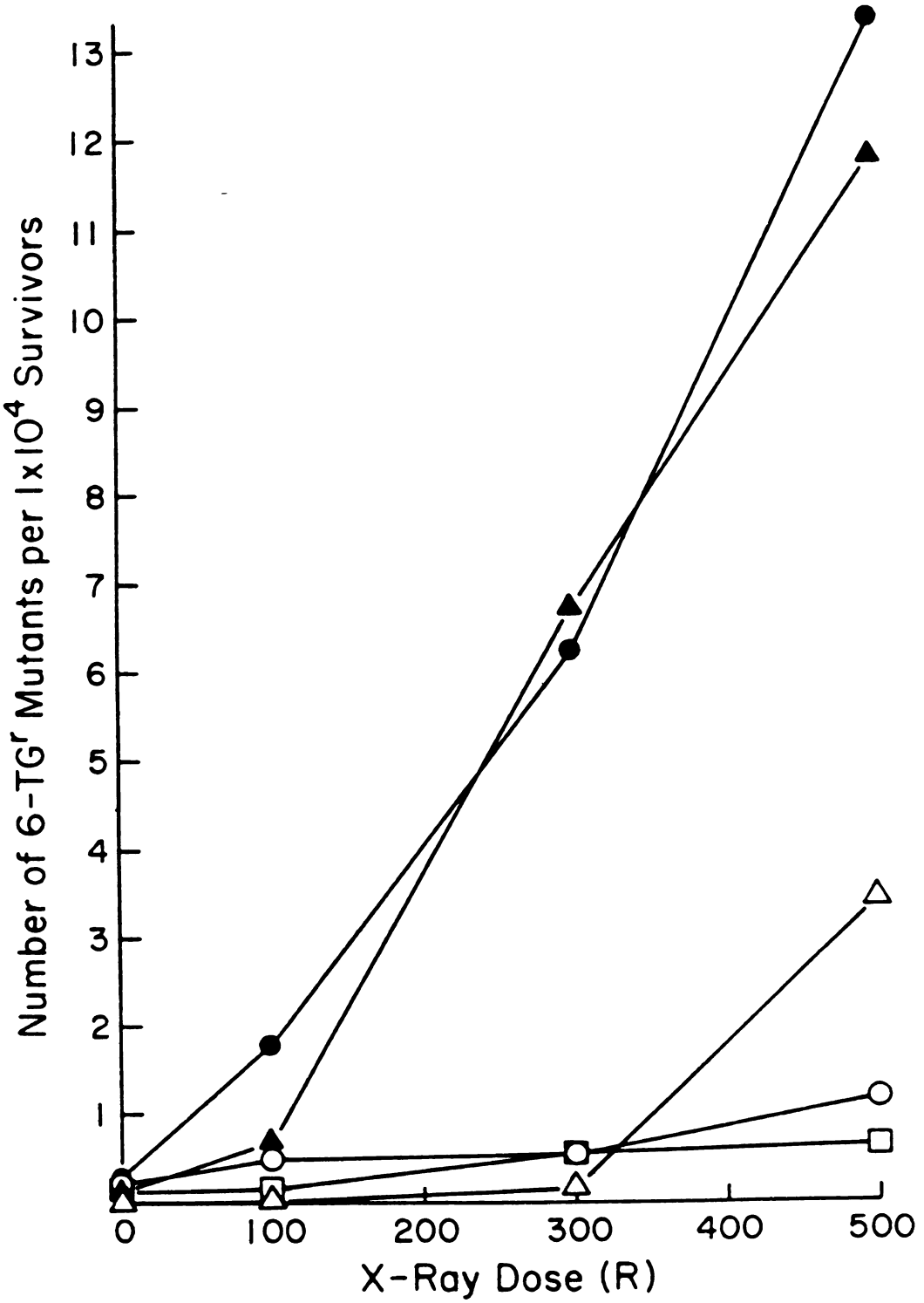

Number of 6-TG^r Mutants per 1x10^4 Survivors
13
12
11
10
9
8
7
6
5
4
3
2
1
0
100
200
300
400
500
X-Ray Dose (R)

Figure 9

than the frequencies observed for the parental cell line or the normal human fibroblasts with same treatments. The results are repeatable in two experiments.

Comparison of ultraviolet light induced mutant frequencies between HTP_3-4 and HTX6TG-1/5AC4-1 cell lines

Ultraviolet (UV) light, another physical mutagen known to induce almost exclusively point mutations and small deletions was chosen to study the mutation inductions in these cell lines. Figure 10 shows that there is no significant difference in UV sensitivity between the two human teratocarcinoma cell lines, HPT_3-4 and HTX6TG-1/5AC4-1. However, the UV-induced cytotoxicity in both HAT^r human teratocarcinoma cell lines is slightly higher than that in normal human cells but is significantly lower than that of xeroderma pigmentosum cells (Glover et al. 1979). Although UV light is a weak mutagen at the various low doses (0-7.5 J/m^2) used to treat these two cell lines, the mutant frequencies of the HTX6TG-1/5AC4-1 cell line were 3-4 fold higher than that of the parental HTP_3-4 cell line (Figure 11). The results were comparable in two separate experiments.

Comparison of MNNG induced mutant frequencies between HTP_3-4 and HTX6TG-1/5AC4-1 cell lines

To test if the HTX6TG-1/5AC4-1 also exhibits higher induced mutations by chemical mutagens, the potent alkylating agent, MNNG, was chosen for this study. The results (Figure

Figure 10. The UV survival curves for two HAT^r huamn teratocarcinoma cell lines. Two separate experiments, designated as circles and triangles, were carried out. Solid circles and triangles stand for results obtained from the HTX6TG-1/5AC4-1 cell line. Open circles and triangles represent results from the parental HTP_3-4 cell line.

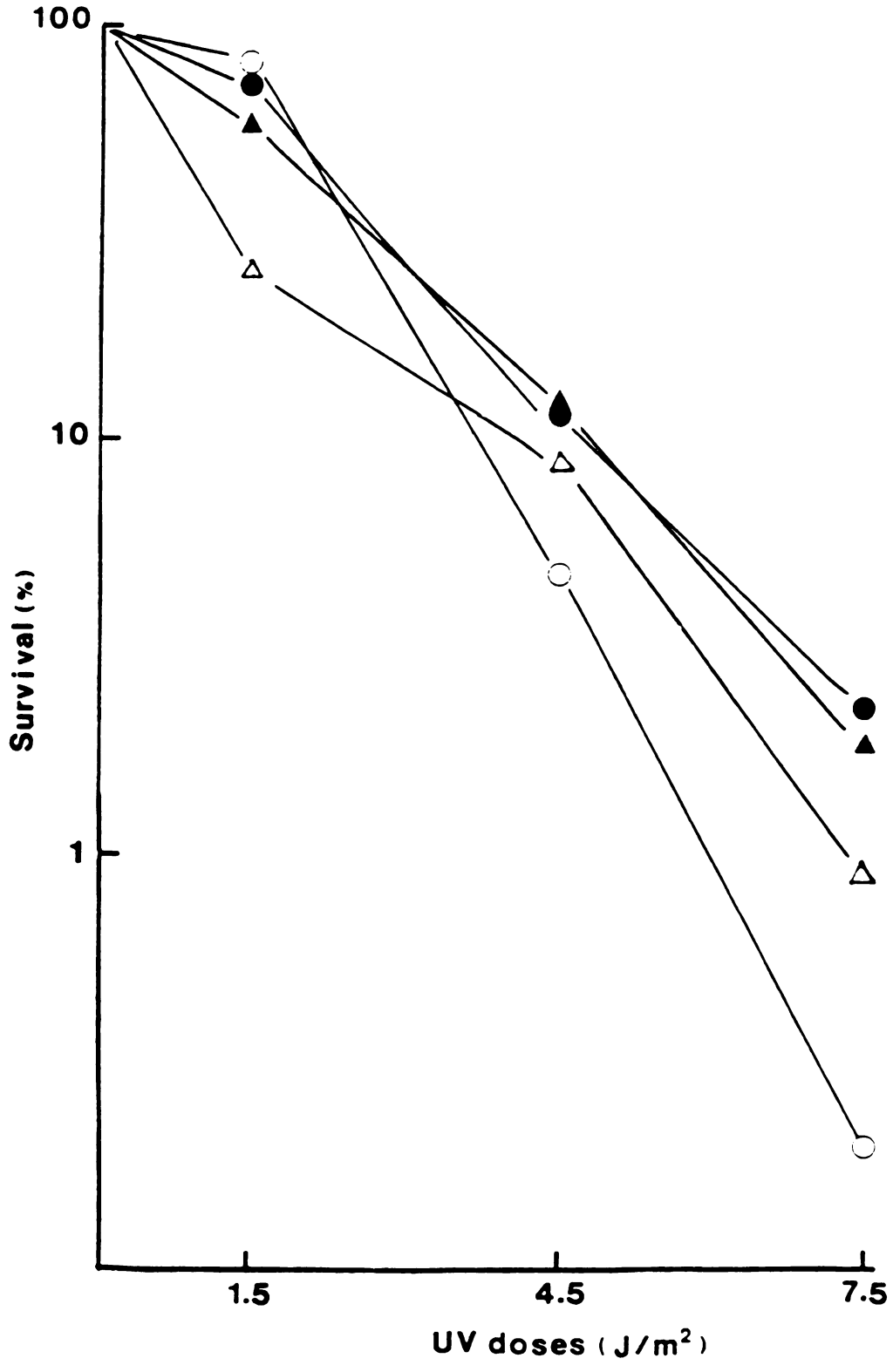


Figure 10

Figure 11. UV-induced 6-TG^r mutant frequencies for the HTX6TG-1/5AC4-1 cell line (solid circles and triangles) and the parental HTP_3-4 cell line (open circles and triangles) from two separate experiments (circles for first experiment and triangles for the second experiment).

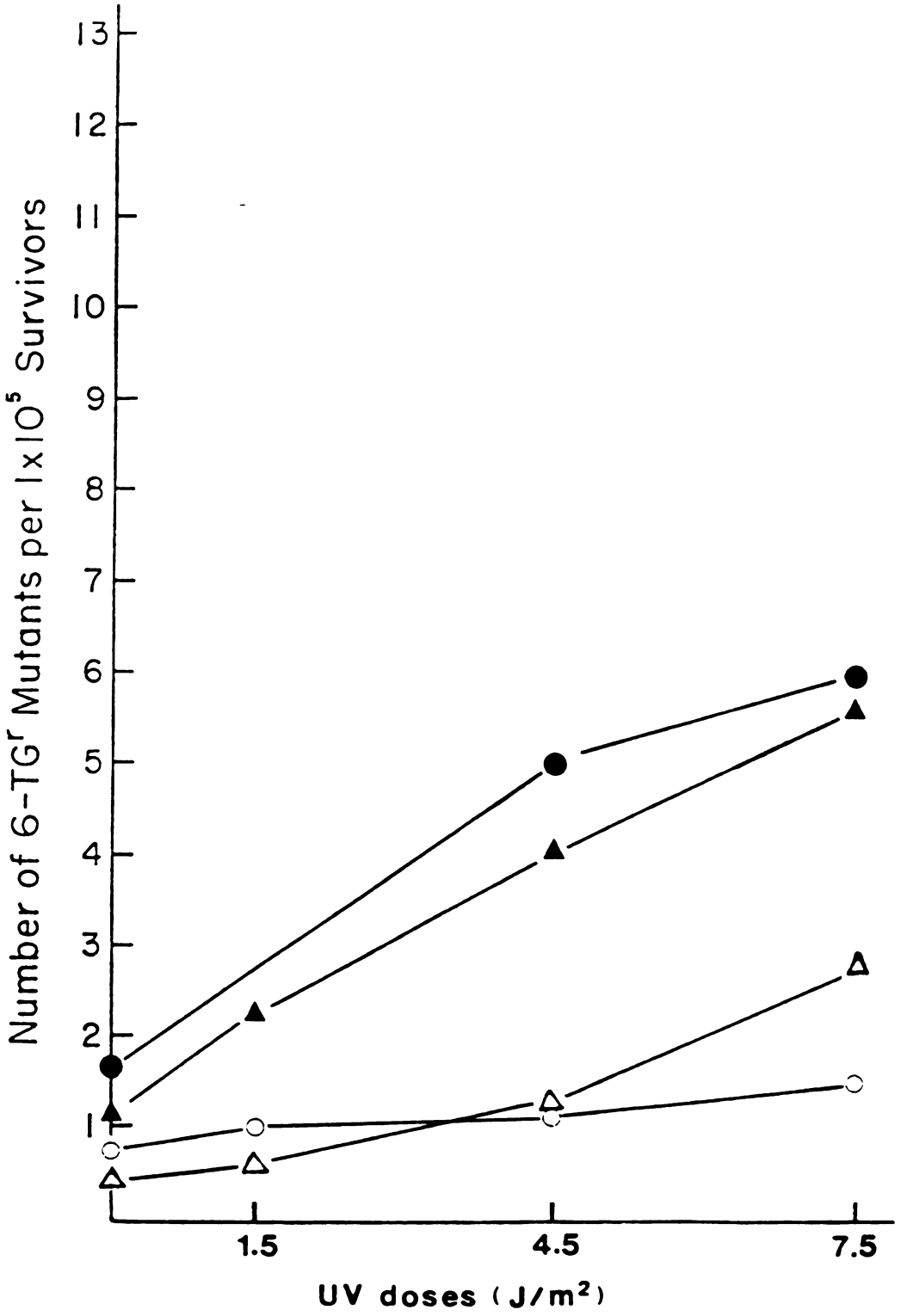

Number of 6-TG^r Mutants per 1x10^5 Survivors
13
12
11
10
9
8
7
6
5
4
3
2
1
1.5
4.5
7.5
UV doses (J/m^2)

Figure 11

12) indicate that cell survival after treatment with various doses (0-10 uM) of MNNG were not significantly different between the HPT_3-4 and HTX6TG-1/5AC4-1 cell lines. The mutant frequencies in the HTX6TG-1/5AC4-1 cell line, however, were increased about 5-50 fold compared to the HTP_3-4 cell line (Figure 13). The results were repeatable in two separate experiments.

Comparison of daunomycin induced mutant frequencies between HTP_3-4 and HTX6TG-1/5AC4-1 cell lines

In order to assess the effect of a different class of chemical mutagens in the new assay system, the clastogen, daunomycin, a well-known chemical with the ability to cause chromosome damages, was chosen to compare its mutagenic activities in the two human cell lines. Results shown in Figure 14 indicate that both HTP_3-4 and HTX6TG-1/5AC4-1 cells had similar cytotoxicity after treatment with various doses (0-75 ng/ml) of daunomycin. However, a significant (5 fold) increase of mutant frequencies was found for HTX6TG-1/5AC4-1 cells after treatments with various doses of daunomycin in two separate experiments as compared to the parental HTP_3-4 cell line (Figure 15). Again, the HTX6TG-1/5AC4-1 cell line was found to be more sensitive to detect a chromosomal mutagen or clastogen.

Molecular characterization of X-ray induced mutations at the hprt locus by the Southern blotting and hybridization

High-molecular weight genomic DNA from different X-ray induced 6-TG^r mutants derived from the HTX6TG-1/5AC4-1 cell

Figure 12. Survival cruves of MNNG for the HTX6TG-1/5AC4-1 cell line (solid circles and triangles) and the parental HTP_3-4 cell line (open circles and triangles) from two separate experiments (circles for first experiment and triangles for the second experiment).

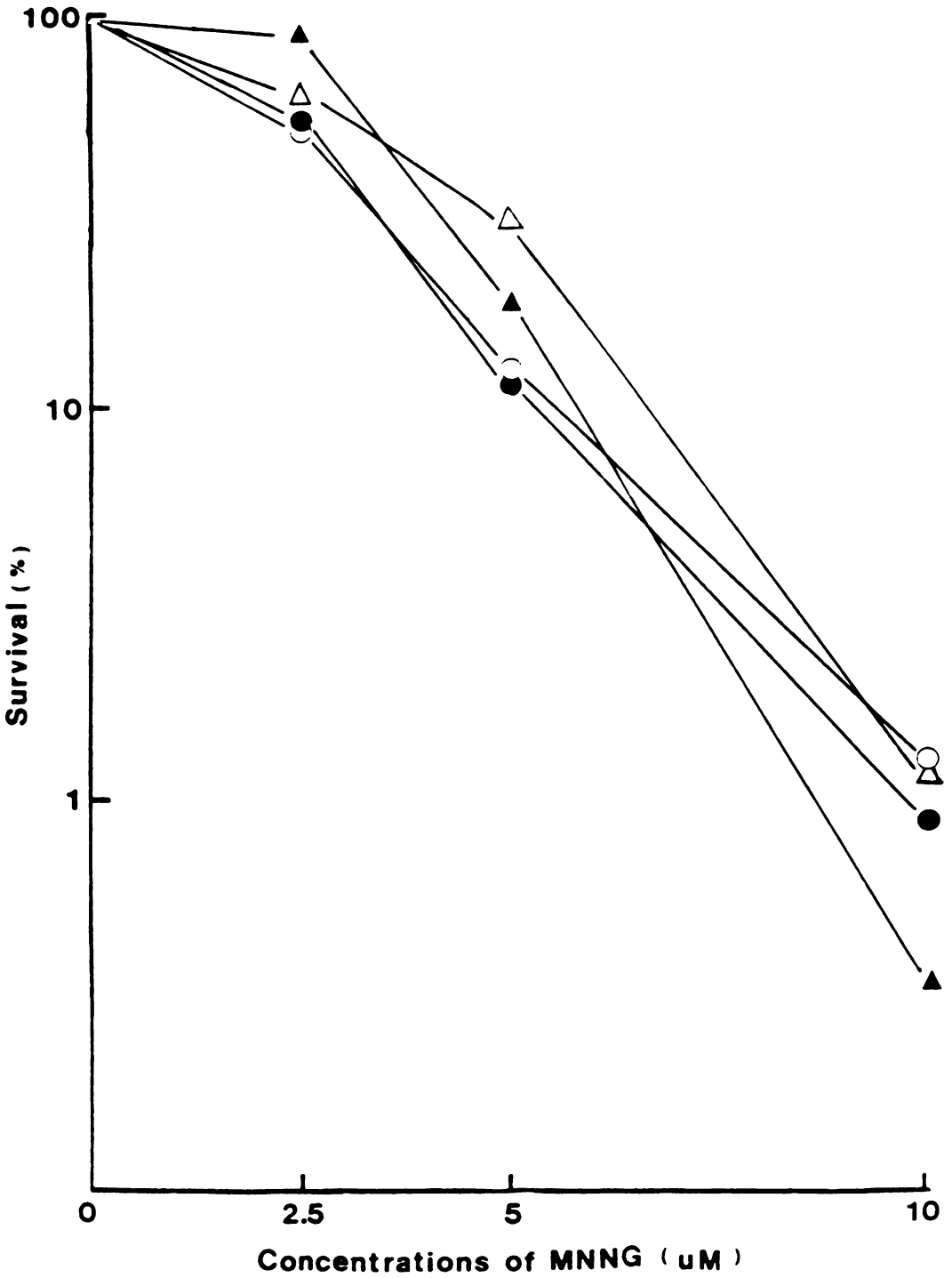


Figure 12

Figure 13. MNNG-induced 6-TG^r mutant frequencies for the HTX6TG-1/5AC4-1 cell line (solid circles and triangles) and the parental HTP_3-4 cell line (open circles and triangles) from two separate experiments. The first experiment is symbolized by circles and the second experiment is symbolized by triangles.

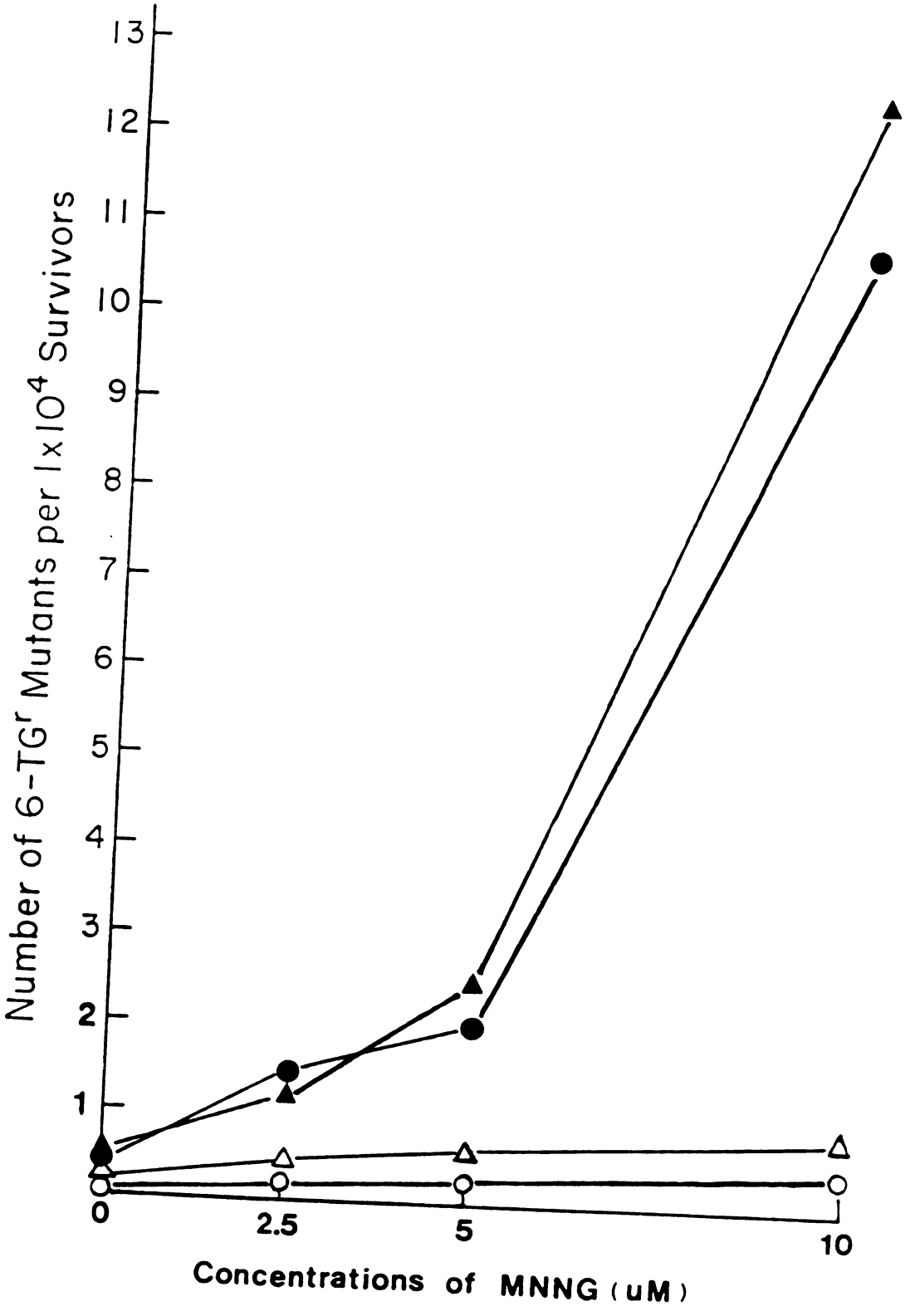

Number of 6-TG^r Mutants per 1x10^4 Survivors
13
12
11
10
9
8
7
6
5
4
3
2
1
0
2.5
5
10
Concentrations of MNNG (uM)

Figure 13

Figure 14. Survival cruves of daunomycin for HTX6TG-1/5AC4-1 cell line (solid circles and triangles) and the parental HTP_3-4 cell line (open circles and triangles) from two separate experiments. The first experiment is symbolized by circles and the second experiment is symbolized by triangles.

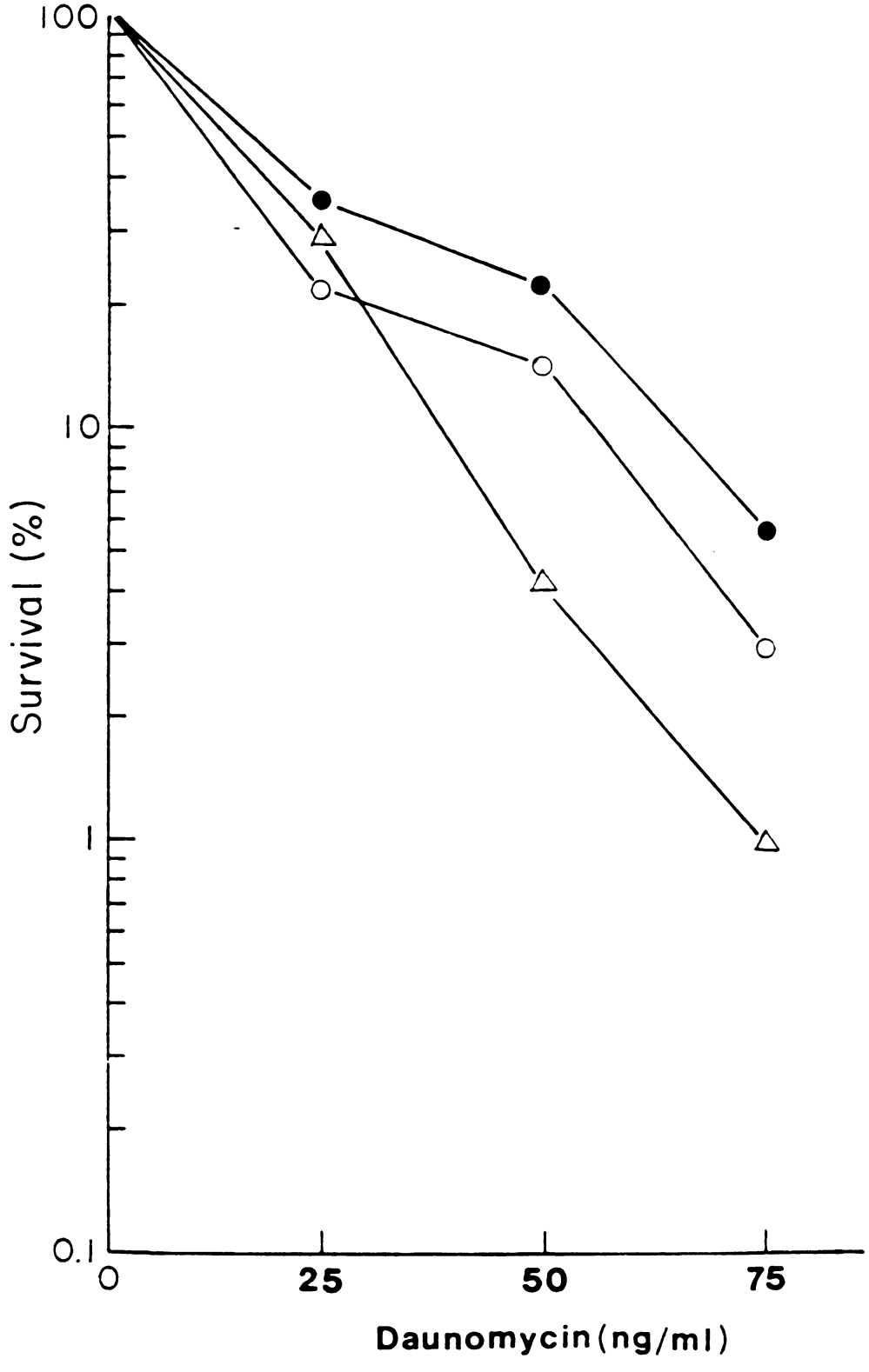


Figure 14

Figure 15. Daunomycin-induced 6-TG^r mutant frequencies for the HTX6TG-1/5AC4-1 cell line (solid circles and triangles) and the parental HTP_3-4 cell line (open circles and triangles) from two separate experiments. The first experiment is symbolized by circles and the second experiment is symbolized by triangles.

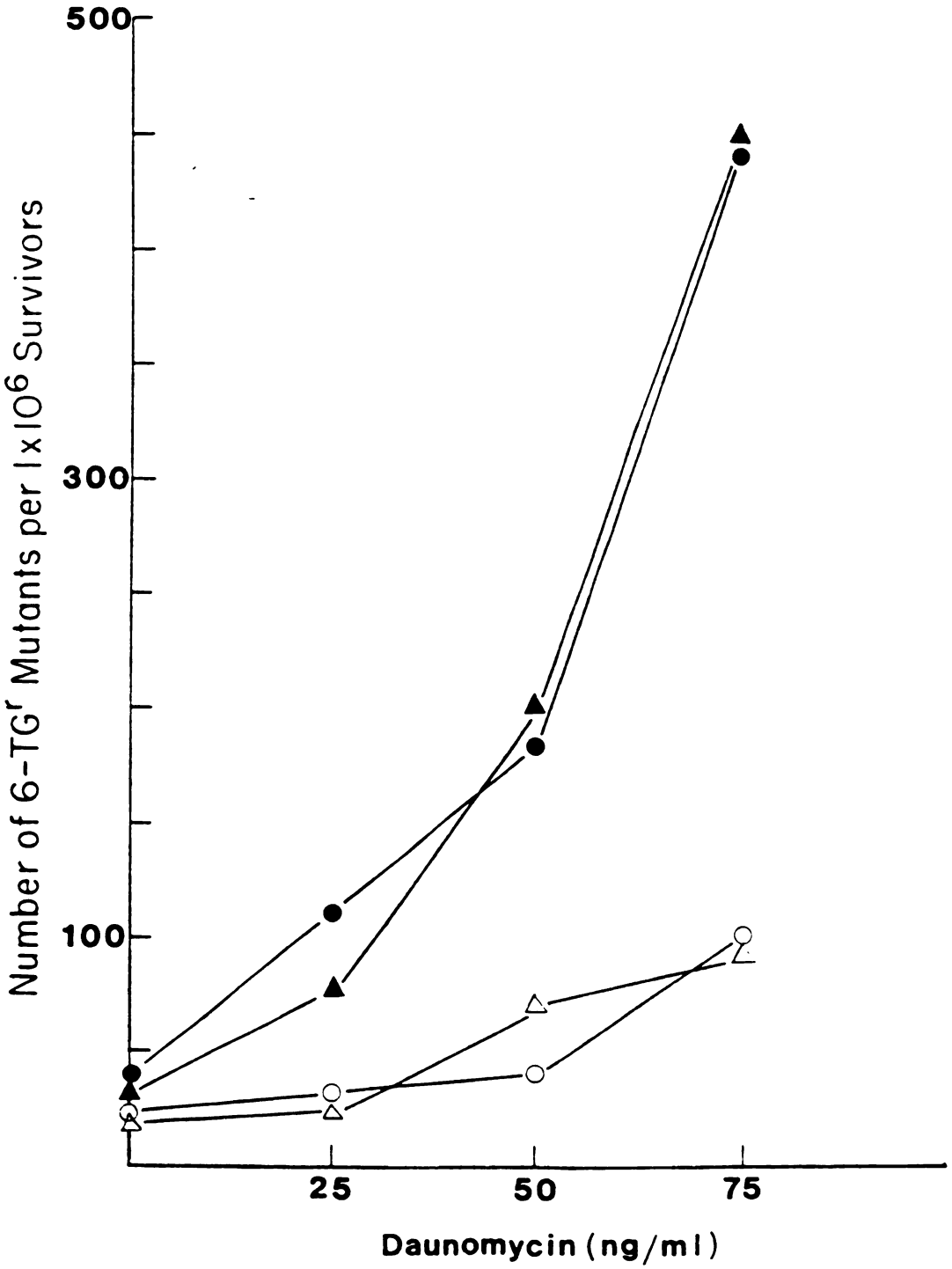

500
300
100
Number of 6-TG^r Mutants per 1x10^6 Survivors
25
50
75
Daunomycin (ng/ml)

Figure 15

line were digested with the restriction endonuclease *PstI* to yield the banding patterns as depicted in Figure 16 after hybridization with a labeled hprt cDNA probe. As observed in previous studies by several investigators (Patel et al. 1984, Nicklas et al. 1989), the *PstI*-digestion of the human hprt gene resulted in autoradiographic bands of 5660 (exon 4), 5405 (exons 5 and 6), 6144 (exons 7 to 9), 3287 (exon 1), 2507 (exon 2) and 1325 (exon 3) base pairs (Figure 16, lanes 11-13). Three cross-hybridizing fragments resulted from the hybridization of the hprt cDNA probe to four nonfunctional hprt pseudogenes located in autosomes of the human genome (Fuscoe et al. 1983). The results shown in Figure 16 reveal that the HTX6TG-1 cell line derived from X-ray irradiated HPT_3-4 cells has an intragenic deletion around exon 7 to 9 region for the hprt gene on the active X chromosome. Therefore, deletion mutants of hprt gene on the inactive chromosome from X-ray irradiated HTX6TG-1/5AC4-1 cells could be identified by the missing band of exons 7 to 9. In addition, genomic DNA isolated from 29 clones of high doses (300 and 500 rads) X-rays-induced 6-TG^r HTX6TG-1/5AC4-1 cells and hybridized with the hprt cDNA probe (Figure 16) showed that 21 clones (72%) are deletion mutants missing the exon 7-9 band. These results further support that ionizing radiation induces predominately deletions/rearrangements at the hprt locus in mammalian and human cells (Vrieling et al. 1985,

Figure 16. Molecular analyses of X-ray induced 6-TG^r mutants from HTX6TG-1/5AC4-1 cells. Genomic DNA were digested with *Pst*I , run on 0.8% agarose gel, and transfered to Hybond™-N nylon membrane. The membrane was hybridized with random priming labeled-hprt cDNA. Lane 1: Molecular weight markers from *Hind*III and *Eco*RI digested lamda DNA; lanes 11, 12 and 13 are *Pst*I-digested genomic DNAs from HTP_3-4, HTX6TG-1 and HTX6TG-1/5AC4-1 cell lines, respectively; Lanes 2-10 are *Pst*I-digested genomic DNAs from different 6TG^r clones of X-ray (500 rads) irradiated HTX6TG-1/5AC4-1 cells; and lanes 14-17 are *Pst*I-digested genomic DNAs from different 6TG^r clones of X-ray (300 rads) irradiated HTX6TG-1/5AC4-1 cells.

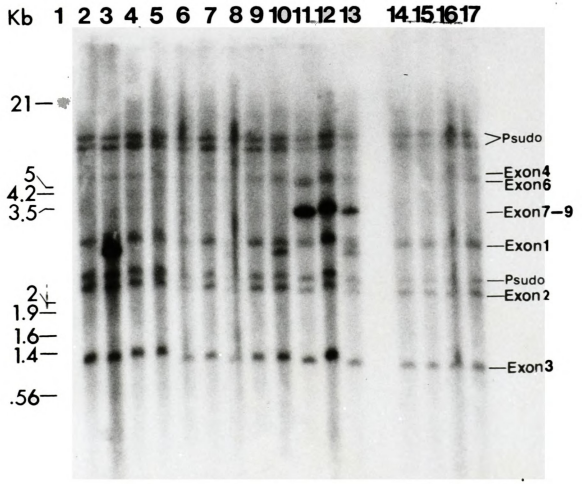

Kb 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
21
5
4.2
3.5
2
1.9
1.6
1.4
.56
Psudo
Exon4
Exon6
Exon7–9
Exon1
Psudo
Exon2
Exon3

Figure 16

Skulimonski et al. 1986, Bradley et al. 1987, Liber et al. 1987, Whaley and Little 1990). The results also indicate that the HTX6TG-1/5AC4-1 cell line, despite its female cell origin, can be used to study deletion mutations at the molecular level.

Molecular characterization of UV-induced mutations at the hprt locus by direct sequencing of PCR-amplified hprt cDNAs

The purpose of this experiment is to determine if the mutagen sensitive HTX6TG-1/5AC4-1 cell line can be used to study hprt gene mutations at the molecular level.

The first strand cDNA copied from mRNA of a 6-TG^r clone was first synthesized by the reverse transcriptase directly from cell lysate of a single colony or 500 cells as DNA template for PCR amplification. The mutated hprt cDNA in the mixture was amplified with two rounds of PCR using two pairs of hprt specific primers. The outside pair of primers (PCR-1 and PCR-2) was first added to the mixture to amplify about 200,000 fold of mutated hprt cDNA with 30 cycles of PCR (Saiki et al. 1985). After the second round of amplification with another pair of primers (PCR-3 and PCR-4) adjacent to the outside primers, the amplification reached 10^{10} to 10^{11}-fold and a quantity of 5 to 10 mg of DNA template was obtained for direct nucleotide sequencing. Twenty-four 6-TG^r clones were isolated from 12 J/m^2 UV-irradiated HTX6TG-1/5AC4-1 cells from four separate experiments. The PCR amplification of mutated hprt cDNA from 500 cells was performed in the Thermocycler.

Figure 17 shows the DNA from 10% of amplification mixture for each sample after two rounds of PCR and electrophoresis in 1.5% ethidium bromide-stained agarose gel. No visible bands were seen in ethidium bromide-stained agarose gel after electrophoresis for eight clones in three separate experiments, presumably due to the deletion of hprt gene or the lack of hprt mRNA in these clones. Sixteen clones with full size hprt cDNA were used for determinations of the hprt cDNA sequence. A representative sequencing gel is shown in Figure 18. The arrow indicates a transversion mutation on 542 base (number 1 base is designated to the "A" in start codon ATG) where T was mutated to A. The mutation presumably changed the amino acid, phenylalanine to tyrosine in the hprt gene and results in the 6-TG^r phenotype. Table 3 summarized the mutations observed for the ten 6-TG^r clones analyzed using the direct nucleotide sequencing of PCR amplified hprt cDNA.

Among the ten clones analyzed, UVE2 and UVE3 clones are from the same experiment and have identical mutations. They are likely to be from the same origin. Similarly UVC3 and UVC7 are also likely to be derived from the same mutation. Therefore, a total of eight hprt cDNA sequences from independent UV-induced 6-TG^r clones have been determined by this study. The analysis of these results indicates that two clones contain single base deletion at base 27. The remaining six clones contain single base substitutions spreading in the entire hprt cDNA. Among them, one G to A transition, one T to

Figure 17. Identification and quantitation of PCR-amplified hprt cDNA on an ethidium bromide-stained 1.5% agarose gel. Lanes 1-5 are samples of two rounds-PCR amplified hprt cDNA from reverse transcribed single-stranded cDNAs from 500 cells' lysate; lane 6 is the sample amplified from 1 pg hprt cDNA positive control; and lane 7 is molecular weight markers from *Hind*III and *Eco*RI digested lamda DNA.

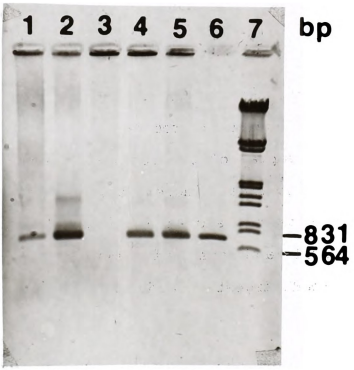


Figure 17

Figure 18. A representative DNA sequencing gel showing a specific change in the hprt cDNA sequence from a 6-TG^r mutant (UVE3) of UV-irradiated HTX6TG-1/5AC4-1 cells. The sequencing reaction was performed with the 5'-end labeled hprt sequencing primer 3 and the chain termination method for double-stranded PCR-amplified hprt cDNA. The T to A transversion in base 542 has altered the amino acid phenylalanine to tyrosine.

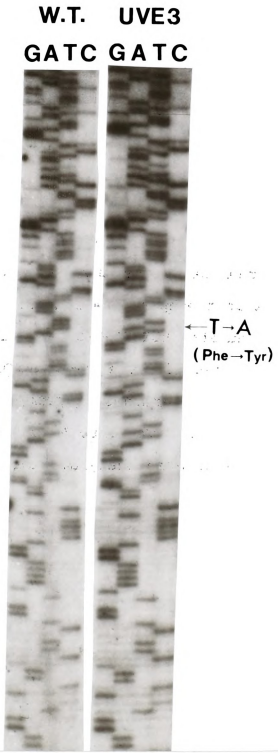


Figure 18

Table 3. Summary of UV-induced 6-TG^r mutants using PCR amplification of reverse transcribed hprt cDNA by direct nucleotide sequencing.

6-TG^r mutants	Target sequences	Mutation sites*	Amino acid changes	Nature of mutation
UVE1	TGGAA	^{400}G-->A	Glu-->Lys	Transition
UVE2&E3	ATTTG	^{542}T-->A	Phe-->Tyr	Transversion
UVE6	TCTTT	^{223}T-->A	Phe-->ILe	Transversion
UVC3&C7	ATTCT	^{221}T-->C	Phe-->Ser	Transition
UVC4	GTGAT	^{27}G deletion	--	Frameshift
UVD2	ATTTT	^{295}T-->G	Phe-->Val	Transversion
UVD6	GTGAT	^{27}G deletion	--	Frameshift
UVD7	TTTGG	^{95}T-->A	Leu-->stop	Transversion

*The cDNA sequence of the hprt gene is based on a previous report (Jolly et al. 1983). The number of nucleotide is started from the initiation codon of translation, AUG in which the "A" is labeled as number 1 nucleotide.

C transition, one T to G transversion and three T to A transversions, all at different base positions. Further analysis of base sequences surrounding the base changes revealed that all of them can be attributable to pyrimidine dimers formed on the coding or complementary strand. Although the sample of mutants analyzed is small, the abundance of T to A transversion in sequences involving thymidine-thymidine dimer is clear. The implications of these results will be discussed later. More importantly, the results clearly show that the mutagen sensitive HT6TG-1/5AC4-1 cell line can be used to study the hprt gene mutations at the molecular level.

DISCUSSION

The major results from this dissertation research are 1) the development of a near diploid human epithelial cell line HTX6TG-1/5AC4-1 which is sensitive to mutation induction at the hprt locus by various mutagens (i.e. X-rays, UV, MNNG and daunomycin); 2) the demonstration that this mutagen sensitive cell line can be used to study deletions and point mutations of the hprt gene at the molecular level; and 3) the substantiation that thymidine-thymidine dimers are mutagenic and that adenine insertion rule may not be valid in UV-induced mutations in this mutagen sensitive cell line.

In human cells, there are few genes besides the hprt gene that can be used for mutation assays. Among them, the Na^+/K^+ ATPase mutant gene (ouabain-resistant mutant) shows dominant or codominant phenotype but the frequency of the induced mutations at this locus is very low (Baker et al. 1979). The others (i.e. the thymidine kinase (tk), the adenine phosphoribosyl transferase (aprt) and the thymidylate synthetase genes) are recessive for mutant phenotypes and located on autosomes. Therefore two mutations are required for the expression of mutant phenotypes. So far, no hemizygous locus has been reported to be created for these genes in a permanent human cell line. The hprt gene remains the best locus for mutation assay in human or mammalian cells due to

the following advantages: 1) the functional hemizygous condition of the gene on the X-chromosome allows the expression of mutant phenotype after the mutation of a single functional gene; 2) the easy selection of mutants with forward and reverse mutation by 6-TG- and HAT-containing medium respectively and 3) the small size of the cDNA is desirable for sequence analysis.

Compared to existing human and mammalian in vitro mutation assays for environmental mutagens, the HTX6TG-1/5AC-1 cell line has the following advantages: 1) the assay using a human epithelial cell line with near normal karyotype [46 XX, t(15;20) and t(11;X)] detects genetic changes relevant to human cell mutations since there is evidence that repair mechanisms in rodent cells are different from human cells (Regan and Setlow 1973). Furthermore, since 90% of fatal malignant tumors are carcinoma arising from different types of epithelial cells (Peto 1977), the choice of an epithelial cell line is better than a cell line of fibroblast origin; 2) the assay is sensitive in detecting a variety of physical and chemical mutagens; 3) the assay can be used to analyze deletion by Southern blotting of genomic DNA and base alterations by sequencing of the hprt cDNA. Therefore the precise mutations can be revealed at the molecular level; and 4) the assay is simple and inexpensive. The permanent cell line can be cultured in a modified Eagle's medium supplemented with low concentration of fetal bovine serum (5%) and has high

colony-forming efficiency (60%). The quantitative mutation assay and the DNA sequencing of mutant hprt gene using PCR amplified cDNA is relatively simple and inexpensive. With these attributes, this mutagen-sensitive cell line should be very useful for determining the mutagenic potential of various environmental agents.

Concerning the origin of sensitivity of the HTX6TG-1/5AC4-1 cell line to various physical and chemical mutagens, the characterization and the effect of the procedure to derive this cell line are consistent with a genotype I initially intended to create, i.e. it has a deleted non-functional hprt gene on the active X-chromosome and a reactivated functional hprt gene on the inactive chromosome. Therefore deletions or mutations of essential genes on the inactive chromosome accompanying the hprt mutation would not affect the survival of the mutant, since homologous genes are present in the active chromosome. The confer of mutagen sensitivity by a similar strategy has been demonstrated in rodent cells as reviewed before (DeMarini et al. 1989). The evidence for this hypothesis are as follows. Firstly, the karyotype analysis of the HTX6TG-1/5AC4-1 cell line revealed that the cell line contains both X-chromosomes. Although one of them is involved in a reciprocal translocation, no visible deletion was found in either chromosome. Therefore, except for the hprt gene, homologous genes are likely to be present in both X-chromosomes. Secondly, the HTX6TG-1/5AC4-1 is derived from a

non-revertible 6-TG^r cell line (HTX6TG-1) after X-rays treatment which is known to induce primarily deletion mutations. The presence of deletion in one hprt gene has been revealed by Southern blotting of genomic DNA for X-ray induced 6-TG^r mutants. Recently, the entire sequence of the 44kb human hprt genomic DNA has been determined (Edwards et al. 1990). The *Pst*I-digested genomic DNA fragments corresponding to the nine exons have been identified. There are six fragments with the size of 1325 (exon 3), 2507 (exon 2), 3287 (exon 1), 4144 (exons 7-9), 5405 (exon 5 and 6) and 5660 (exon 4) base pair. A high frequency of X-ray induced 6-TG^r mutants was found to lost the 4144 band in *Pst*I-digested genomic DNA as probed by the labeled hprt cDNA. The high frequency of the loss of the exon 7 to 9 band in these mutants is not possible if the whole hprt gene is intact in both X-chromosomes. Since 5-azacytidine is not known to induce deletion mutations, the deletion is most likely induced by X-rays. This X-ray induced deletion of hprt gene is more likely to be on the inactive chromosome rather than on the inactive chromosome since the latter does not create a 6-TG^r mutant. Thirdly, the HAT-resistant phenotype (presence of functional hprt gene) of the HTX6TG-1/5AC4-1 cell line was recovered after its parental 6-TG^r cells (HTX6TG-1) (absence of functional hprt gene) was treated with 5-azacytidine, an agent known to reactivate inactive gene by epigenetic mechanism (i.e. hypomethylation of genes) rather than reverse mutations. This effects of 5-azacytidine have

been reported by several investigators including the reactivation of hprt genes on the inactive X-chromosome in human and rodent cells (Mohandas et al. 1981, Graves 1982; Jones et al. 1982; Pasterno et al. 1985; Ivarie and Morris 1986; and Hockey et al. 1989). The nonmutagenic activity of 5-azacytidine has also been documented for mammalian cells (Landolph and Jones 1982; Kerbel et al. 1984).

Alternatively, one might argue that the mutagen-sensitive phenotype is due to an abnormal error-prone DNA repair mechanism induced in these cells. Indeed there is a report that 5-azacytidine was able to reactivate a DNA repair gene in CHO cells (Jeggo and Holliday 1986). However, this presupposes that the 5-azacytidine simultaneously reactivates a hprt gene and the DNA repair gene. Although possibility cannot be ruled out at this time, the probability for its occurrence has to be considered as small.

There are few mechanisms that can be utilized to improve the sensitivity of a mutation assay. Two of them have been mentioned, i.e. 1) the use of a target gene on a non-essential chromosome or on a chromosome which has a homologous chromosome carrying identical genes (usually the autosomes) (see review by DeMarini et al. 1989) and 2) the use of error-prone DNA repair mutants. For instance, in prokaryotes, an error-prone plasmid pKM101 has been introduced into the Salmonella Typhimurium to maximize the mutant frequencies. In eukaryotes, mutant cells defective in DNA repair were found to

have higher induced mutation frequencies (see review by Bohr et al. 1989). In addition, the sensitivity of an assay could also be enhanced by increasing the ability of the target cells to metabolically activate promutagens/ procarcinogens (Crespi et al. 1989).

Most HAT^r clones derived from the HTX6TG-1 cell lines, including the HTX6TG-1/5AC4 cell line, after 5-azacytidine treatment were unstable with high spontaneous reversion frequencies. This indicates that the methylation status of these reactivated clones are not stable and readily revertible. The stable HTX6TG-1/5AC4-1 cell line was recovered after subcloning and was found to contain a reciprocal translocation near the Xq27 band where the hprt gene is located. Therefore the t(11;X) translocation is suspected to play a role in stabilizing the reactivated hprt gene. To prove this hypothesis it is necessary to demonstrate that the functional hprt gene is, indeed, on the t(11;X) chromosome. This can be done by somatic cell hybridization studies.

X-rays were found to induce significantly higher frequencies of $6\text{-}TG^r$ mutants in the HTX6TG-1/5AC4-1 cell line than in its parental cell line or normal human fibroblasts. Furthermore, consistent with other reports, X-rays induced mutants were found to contain mostly deletions in the hprt genes. The different X-ray induced mutant frequencies in these cell lines may be explained by the differential recovery of 6-

TG^r mutants with deletions of essential genes on the same chromosome.

MNNG is a direct potent mutagen/carcinogen which induces point mutations through formation of mutagenic 0^6-alkylguanine DNA adducts in prokaryotes and eukaryotes (Peterson et al. 1978; Meuth 1981). The differential recovery of MNNG-induced mutants in the HTX6TG-1/5AC-1 and its parental cell lines may be attributable to differential recovery of 6-TG^r mutants with simultaneous mutations of essential genes on the same chromosome. As was observed in X-rays induced mutants, the differential recovery was more striking at higher MNNG doses. A curvilinear dose response was evident for both mutagens. This casts doubt upon the validity of assessing the effects of low doses from extrapolation of results obtained with high doses, a current practice in in vivo and in vitro studies.

In order to test whether the HTX6TG-1 cell line is also sensitive in detecting chemical mutagens/carcinogens with different mechanisms, daunomycin, a clastogen, was included in this study. It has been shown that daunomycin interacts with topoisomerase II and was extensively used in the treatment of human leukemias and lymphomas (Wiernik 1980). The primary action of the daunomycin is generally thought to be due to its ability to interfere with the breakage-reunion reaction catalyzed by topoisomerase II by trapping a reaction intermediate, termed the "cleavage complex" (D'Arpa and Liu 1989, Zhang et al. 1990). The trapped "cleavage complexes" are

"DNA-protein complexes" containing duplex DNA fragments whose 5'-phosphoryl terminal are each covalently linked to a topoisomerase II molecule through a phosphotyrosine bond. These trapped cleavage complexes would be viewed as a bulky DNA adducts which generate double- and single-strand DNA breaks and was originally found in topoisomerase effectors-treated culture mammalian cells (Loike and Horwitz 1976, Ross et al. 1978, Long and Minocha 1981). The trapped cleavage complexes are presumed to be the initial event that triggers rapid cell death and other cellular responses, such as G_2 arrest and an increased rate of sister chromatid exchange. The daunomycin has been designated as weak or non-mutagenic in CHO/hprt and sister chromatid exchanges/CHO systems (Singh and Gupta 1983, Li et al. 1988). However, the in vitro tumorigenicity of the compound to induce mammary and renal tumors in rats with single I.V. doses were comparable to that of radiations (Sternberg et al. 1972, Shellabarger et al. 1960). Furthermore, studies of in vitro transformation of mouse M2 fibroblasts indicated that the transforming activity of daunomycin was comparable to that of the potent carcinogen, MNNG (Marquardt et al. 1976). The results presented in Figure 15 suggest that daunomycin is mutagenic in the HTX6TG-1/5AC4-1 cell line. This mutagenic activity may be underlying its in vivo tumorigenicity and the in vitro transforming activity in mouse M2 fibroblasts.

From experimental mutagenesis, UV was generally found to be a weak or moderate but not a potent mutagen and capable of inducing various types of mutations such as base substitutions, frameshift mutations and deletions in L5178Y/TK$^{+/-}$ cells and human TK6 cells (Yandell et al. 1986, Little et al. 1987). Therefore UV was chosen not only for the mutagenesis study but for direct nucleotide sequencing of PCR amplified hprt cDNAs from independently derived 6-TG^r mutants. The results in Figure 11 show that the frequencies of UV-induced 6-TG^r mutants were four times higher in the HTX6TG-1/5AC4-1 cells than in the parental HPT$_3$-4 cells. Although the difference was not as striking as those found in X-rays or MNNG-induced mutations, it shows that the new assay is sensitive in detecting weak as well as potent mutagens.

The results from sequencing studies as listed in Table 3 indicate that the HTX6TG-1/5AC4-1 cell line, which is sensitive in detecting mutations, is suitable for the analysis of the nature of mutations by direct nucleotide sequencing of PCR amplified hprt cDNA.

The nature of molecular alterations in 10 UV-induced 6-TG^r mutant clones as revealed by the DNA sequencing was listed in Table 3. The results indicate that UV induced base substitutions as well as base deletions. An analysis of the six independent base substitution mutations reveals that half of them (3/6) are T·A to A·T transversions. The remaining

mutations are T to G transversion, G to A transition and T to C transition (one each).

All of these mutations are attributable to potential pyrimidine dimers formed on the sense or complementary DNA strand similar to results obtained by other investigators (see reviews by Meuth 1990, Skandalis and Glickman 1990). In contrast to previous studies of UV-induced mutations in shuttle vectors within human cells (Bredberg et al. 1986, Brash et al. 1987; Wang et al. 1991) which showed that most UV-induced mutations are G·C to A·T transitions conforming to the adenine insertion rule [i.e. mutations resulted from the incorporation of a dAMP opposite the cytosine base of a pyrimidine dimer (C-T or C-C) during DNA replication], the present results indicate that most of the mutations (3/6) arose from misincorporation of a dTMP oppositing a thymine dimer in the coding strand in contrast to previous view that the premutagenic sites are mostly 6-4 photoproducts which are formed preferentially at TC sites (Lippke et al. 1981) (see review by Mitchell 1988). The difference could be due to the location of target genes or due to different repair systems. Since a recent study of UV-induced mutations in human cells indicates that the frequency of UV-induced G·C to A·T transitions was lower in xeroderma pigmentosum variant cells than that in normal cells (Wang et al. 1991).

Whether adenine insertion rule is invalid or not prevailing in HTX6TG-1/5AC4-1 cells still needs to be

substantiated by analysis of more mutants. The ability of thymine dimers to induce mutations in human cells, however, appears certain.

To simplify the procedure for analyzing molecular nature of mutations, I have adopted the technique of polymerase chain reaction (PCR) and direct sequencing of double-stranded PCR products from reverse transcribed hprt cDNA in cell lysate from single colony or small number of cells (Yang et al. 1989). Despite the large 44 Kb of hprt genomic DNA and 1.5 Kb of mRNA, there are only 675 bases of hprt mRNA translated into protein. The 675 bp of hprt gene could be amplified 10^{10}-10^{11} fold from two pairs of primers after reverse transcribed cDNAs in cell lysate. Approximately 500 cells of 6-TG^r mutant cells were lysed for direct nucleotide sequencing to avoid the polymerization errors by the thermostable Taq polymerase in amplification of hprt cDNA. This procedure can produce 5 to 10 mg DNA of the hprt gene for direct sequencing of the coding region without the need for RNA purification or genomic DNA isolation.

Several precautions for this technique need to be mentioned: 1) because polymerases used in this technique can themselves introduce mutations, more than 100 cells of each 6-TG^r mutant clone should be used for reverse transcription and PCR amplification. It is known that the fidelity of M-MLV reverse transcriptase is less than $1x10^{-4}$ (Preston et al. 1988) and the fidelity of the thermostable Taq polymerase is

estimated to be about $2x10^{-4}$ (Saiki et al. 1988). Therefore, to avoid the polymerization error from polymerases, approximately 500 cells from a clone usually were lysed to investigate the mutagen-induced mutation; (2) since the reverse transcribed cDNAs from cell lysate are the starting materials for PCR amplification, a strict requirement of RNase-free conditions is essential in the synthesis of the first-strand cDNAs; (3) due to the high efficiency of PCR amplification, a negative control, starting from the reverse transcription of cDNAs, must be included through the entire procedure to avoid cross-contamination of samples; (4) separate positive controls using 1 pg of hprt cDNA in linearized plasmid in two different rounds of PCR amplification should be included to check the amplification efficiency by two pairs of hprt PCR primers; (5) the agarose gel electrophoresis should be performed to identify and quantitate the PCR amplified hprt cDNA before the direct nucleotide sequencing; and (6) three sequencing primers are necessary to determine the entire hprt sequence with two loading of sequencing sample in 6% 7M urea/polyacrylamide gel. The 5'-end labeling primers should have specific activities higher than $2x10^{6}$ cpm/pmole in order to obtain clear banding from the autoradiography of sequencing gel.

In conclusion, this dissertation research has resulted in the development of a human epithelial cell line with near normal karyotype. This cell line has been shown to be more

sensitive in detecting mutations induced by various physical and chemical mutations. The mutagen sensitivity of the cell line is probably due to the location of the functional target gene (the hprt gene) on an inactive X-chromosome. The cell line is not only useful for determining the mutagenic potential of various environmental agents but also can be used to study the nature of mutations at the molecular level by direct sequencing of the PCR amplified cDNA of the target gene.

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