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Genotoxicity of the Space Environment

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

DOCTOR OF PHILOSOPHY

in the Department of Biology

We accept this dissertation as conforming
to the required standard

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ABSTRACT

This thesis presents a study on possible genetic consequences of the exposure to the space environment during space missions. The present study was undertaken in co-operation with the Canadian Space Agency, and involved the analysis of the lymphocyte samples taken from experienced cosmonauts and trainees. For the analysis of genotoxicity of the space environment, a T-lymphocyte *hprt* clonal assay has been employed. In order to distinguish between artefacts associated with this method and the spaceflight-related effects, we have conducted a series of *in vitro* reconstruction experiments. In these experiments we have analysed interactions between plating efficiency (PE) of T-lymphocytes and efficiency of mutant recovery. Using 12 pairs of independent wild type (WT) and mutant clones, we have demonstrated an inverse correlation between initial viability of the WT cells and survival of mutant cells ($r = 0.3496$, $p < 0.05$). Our data suggest that the presence of WT cells in the selection plates does suppress the recovery of mutants in *HPRT* assay. This effect is stronger in samples with high PE, and may be a source of large error in estimation of mutant frequencies (approx. 3-fold in the range of PEs from 10% to 60%), which is especially relevant when samples with different PEs are compared.

Analysis of samples from cosmonauts was conducted in two experiments. The first experiment involved 5 samples taken in 1992 from cosmonauts who have completed spaceflights ranging in duration from 7 to 365 days. *Hprt* mutant frequencies (MF) in these samples were 2.5-5 times higher than the age-corrected values for healthy, unexposed subjects in Western countries (Tates *et al.*, 1991; Branda *et al.*, 1993), and 2-3-fold higher than those determined for unexposed individuals residing in Russia (Jones *et al.*, 1995). The cosmonaut mutational spectrum differed from that of unexposed healthy subjects ($p=0.042$), and showed a higher incidence of splicing errors, frameshifts, and complex mutations. Distribution of base substitutions was remarkably similar to that observed in Russian twins sampled at the same period (Curry *et al.*, 1998), thus suggestive of possible environmental, diet, or life-style related exposures.

The second study was conducted on samples taken 5 years later and involved trainees and a group of cosmonauts with more uniform (at least 6 months) and recent flight experience. *Hprt* MFs in both cosmonaut and trainee groups were virtually identical (17.2 ± 0.6 and 17.6 ± 4.7 x 10^{-6} respectively), and approximately 2-fold higher than in matching Western controls, although considerably lower than in our previous observations. Mutational spectra in both datasets were very similar to that observed in our earlier study, and were significantly different from spontaneous data (p =0.031-0.038). Distribution of base substitutions, however, did not show any differences.

Our data indicate that the space environment is not genotoxic at the *hprt* locus. At the same time, uniformly high MFs observed in all studied groups suggest that the level of the mutagenic burden in at least megalopolis areas of Russia may be considerably larger than in the West. Also, there are some indications of a possible restructuring of mutagenic burden in post-transitional Russia.

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List of Abbreviations

5'-GMP	5'-guanosine monophosphate
5'-IMP	5-inosine monophosphate
6TG	5-thioguanine
ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
AMSA	amsacrine
Aprt	adenine phosphoribosyltransferase
B-DNA	right-handed DNA helix
cDNA	coding DNA
CE	cloning efficiency
CHO	Chinese hamster ovary cells
CHO A_L	CHO cells carrying Human chromosome
DHFR	dihydrofolate reductase
DMSO	Dimethylsulfoxide
DSB	double-strand break
GCR	galactic cosmic radiation
HLA-A	human lymphocyte antigen A
Hprt	hypoxanthine hosphoribosyltransferase
HZE	particles (high energy + high atomic number)
IL-2	interleukin 2
LET	Linear Energy Transfer
LN	Lesch-Nyhan syndrome
LOH	loss of heterozygocity
MF	mutant frequency
MLA	mouse lymphoma assay
Na/K-ATPase	Na+/K+ adenosine triphosphatase
NG	Normally growing colonies
PE	plating efficiency
PCR	polymerase chain reaction
PRPP	5'-phosphoribosyl-1-pyrophosphate
RBE	relative biological effectiveness
RFLP	restriction fragment length polymorphism
RPMI-1640	Rosewell Park Memorial Institute Medium No. 1640
SB	Southern blots
SCEs	sister chromatid exchanges
SG	slowly growing colonies
SSB	single-strand breaks
SV40	simian virus 40
RT	reverse transcriptase
TG^R	thioguanine resistant
TIMBER	triplex interference mapping by binding element replacement
TK (tk)	thymidine kinase
VM-26	teniposide
VP-16	etoposide
WC	Western control
WT	wild type
Z-DNA	left-handed DNA helix

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Introduction and Thesis Rationale

The era of space exploration that started only four decades ago is one of the milestones of human civilization. The former Soviet Union successfully launched Sputnik I, the world's first artificial satellite, on October 4, 1957. This satellite was small (the size of a basketball, weighing 18.3 pounds), but it marked the start of the space age, and triggered a chain of fundamental political, military, technological, and scientific developments. One month later, on November 3, Sputnik II was launched, this time carrying the first living being, a dog named Laika. These two sensational events concluded the first phase of the space race between the USA and the USSR, and led American Congress to the creation of the National Aeronautics and Space Administration (NASA) in July 1958 (National Aeronautics and Space Act, 1958).

The first attempts were meant to explore the mere technological and biological possibility of entering and surviving the space environment. Further projects demonstrated the tremendous potential of space-based research to expand our knowledge about the universe and ourselves, and in many ways to benefit mankind. Weather forecasting has undergone a revolution because of geostationary meteorological satellites. Satellite communications generated billions of dollars annually, and, more importantly, drastically changed our lives. Earth monitoring satellites (Landsats) have enabled nations to inventory fields of different crops in a fraction of the time this task would require by other means. Similarly, sea monitoring satellites (Seasats) perform a global monitoring of oceans. Global space industry revenues approached $77 billion in 1996, and for the first time surpassed government expenditures. With an annual projected growth rate of over 20%,

total industry revenues are expected to exceed $100 billion before the year 2000 (Mulville, 1998).

Future plans include a wide range of projects focusing on biomedical, technological, and fundamental research, which in some cases require manned space missions. The construction of the International Space Station (ISS), which is a product of the joint efforts of 15 countries, is now underway. Moreover, long-range NASA plans predict a significant increase in space traffic, including transportation, construction of production units, and even tourism (Mulville, 1998).

This anticipated increase in the number and duration of manned space missions strengthens the need for an analysis of the impact of the space environment on human health. Weightlessness and ionizing radiation are two major physical factors affecting the performance and health of astronauts. The physiological consequences of microgravity are well known and have been extensively studied. They include disregulation of the cardio-vascular system, muscle atrophy, disturbances in calcium homeostasis, and motion sickness, *etc*. Exposure to ionizing radiation does not result in obvious immediate effects. However, although the intensity of radiation in space is low, long-term exposure to radiation during extended missions may result in enhanced mutagenesis and increased risk for development of diseases with a genetic component.

Both early and late effects of ionizing radiation in the space environment have been the subject of extensive research for many years (for a review see "Radiation Hazards to Crews of Interplanetary Missions", 1996). However, in the absence of direct data, the risk estimates have mostly been based on information from other sources, including *in vitro* experiments, animal studies, and human studies from occupationally or medically exposed

groups, as well as the atomic bomb survivors in Japan. The unique composition and fluctuations of space radiation are not reflected in these studies, thus introducing a potentially large (400%-1500%) error in the risk estimates (Curtis *et al.*, 1995).

In order to overcome these limitations and to evaluate the actual genotoxicity of the space environment, it is necessary to conduct a direct measurement and analysis of mutation in experienced astronauts. Among the currently available *in vivo* methods, only the HPRT assay allows the analysis of both mutant accumulation and the molecular nature of mutations. This dissertation presents a study of genotoxicity of the space environment using T-lymphocyte samples from experienced cosmonauts and trainees, using the *hprt* gene as a target for molecular analysis. Chapter I presents background information on the nature of ionizing radiation; mutagenicity and mutational specificity of ionizing radiation in different assays with regard to their sensitivity and ability do detect various classes of mutations; ionizing radiation in space; and advantages and limitations of *hprt* as the genetic target. Chapter II deals with the analysis of *hprt* assay-specific biases preventing an accurate measurement of mutant induction after exposure to mutagens. Chapters III and IV describe the results obtained from analysis of samples from Russian trainees and experienced cosmonauts, and, finally, Chapter V contains a discussion on the meaning and importance of our findings.

CHAPTER I – Background

1 Ionizing Radiation

1.1 Introduction

The accumulation of mutations in genomes of eucaryotic organisms, and the subsequent malfunction of affected pathways is very complex. Mutation reflects the result of numerous processes, including I) interaction of both exogenous and endogenous damaging agents with DNA; ii) the formation of a wide spectrum of DNA lesions; iii) the reparability of these lesions depending on their type, location, and sequence context; iv) the correlation of damage with cytotoxicity *vs.* mutagenicity; v) the induction of apoptosis resulting in the specific loss of selected damaged cells), and, vi) the compatibility of introduced mutations with viability which also may depend on the cell type.

Ionizing radiation drew attention almost 70 years ago as the first known mutagen (Muller, 1927). The relevance of ionizing radiation as an environmentally important mutagen has grown since due to the use of radioactive materials in warfare, industry and medicine, and the ever-increasing volume of radioactive waste. This, in addition to occupational and medical exposures, creates the possibility of accidental or intentional massive exposures to ionizing radiation. Unfortunately, recent history provides us with several examples of accidents leading to the contamination of large areas and affecting large groups of people. The most serious of these is the disaster in Chernobyl that not only made substantial parts of Ukraine and Byelorussia unsuitable for living, but also aggravated the environmental situation in Eastern and Central Europe due to radioactive fallout (Pohl-Rüling *et al.*, 1990, 1991).

Radioactive contamination is but a part of a general trend in the modern world. Unlike earlier periods of human history, our current technocratic civilization creates new environmental situations that affect all living beings, not just humans. Large number of potentially genotoxic chemical or physical agents that are produced by our highly industrialized societies have become part of our everyday environment. This creates unprecedented challenges for DNA repair systems due to the higher accumulation rates of DNA damage. An accurate evaluation of potential risks associated with exposure to chemical and physical agents is therefore vitally important and directly related to issue of human health.

Neither the short nor long term genetic consequences of exposure to ionizing radiation are yet clearly defined. In part this reflects the fact that until relatively recently, reliable assays for monitoring process of mutagenesis in mammals have not been available. The employment of mammalian cell systems for study of mutagenesis became possible in the late 50's after the development of methods for the culturing of cells *in vitro*, and the selection for mutations became possible (Chu and Malling, 1968). Since the initial mutagenesis experiments in mammalian cells at the hypoxanthine-phosphoribosyltransferase (*hprt)* locus using 8-azaguanine as the selective agent (Chu and Malling, 1968), a variety of mammalian specific-locus assays have been developed (Sankaranarayanan, 1991). These include adenine phosphoribosyltransferase (*aprt*), thymidine kinase (*tk*), Na+/K+ adenosine triphosphatase (Na+/K+-ATPase), HLA-A, dihydrofolate reductase (DHFR).

Of the established mammalian mutational assays, the *hprt* T-cell assay gene is the most extensively used biomarker of exposure to mutagens both *in vivo* and *in vitro*

(Albertini *et al.*, 1993). As *hprt* is an X-linked gene (only one copy is present in males) selection is relatively easy. In females the situation is similar, as the gene is functionally hemizygous. The only complication in females is that the second copy may complicate molecular analysis. Several studies have shown that exposure to ionizing radiation results in the accumulation of mutations in *hprt* in humans (Albertini *et al.*, 1992). These data contributed substantially to current risk estimates related to such exposures. At the same time, the hemizygous nature of *hprt* (which is not characteristic for the vast majority of genetic loci in genome) raises the question of how accurately (qualitatively and quantitatively) data derived from human mutagenicity studies using *hprt* as a genetic endpoint reflect true relationships between exposure to mutagen and mutagenic response in mammalian cells. Therefore, in order to clarify possible limitations of the *hprt* locus as a genetic endpoint we attempted to compare *in vitro hprt* data with results obtained from other assays employing somatic genes and not directly applicable to human biomonitoring studies (section 3).

1.2 Biological Effects of Ionizing Radiation

Exposure to ionizing radiation results in complex DNA damage including single- and double-strand breaks (dsbs and ssbs) and base modifications. Ionization of molecules occurs upon the impact of photons (X- and γ-rays) or particles (electrons, neutrons and ions of high atomic weight) possessing energy exceeding the ionization potential of the molecules. Upon traversing through the matter and interacting with electrons particles lose energy. This process is described as Linear Energy Transfer (LET) which (for the particles) is described by the Bethe equation:

$$-dE/dX = A \times (z2 \times M \times Ne)/E \times B \times C$$

where A is a constant, z – is the effective charge of the particle, M – is the mass of the particle, E – is the energy of the particle, Ne – is the density of electrons in the interacting matter, B – is the "stopping factor", and C – is the relativistic term. This equation indicates that LET is directly proportional to the charge, molecular weight and density of the matter, and inversely proportional to the energy of the particle.

The mechanism of ionization differs, depending on its source: charged particles cause ionization by snatching electrons from the molecules. After that, their charge decreases, and eventually they may become neutral; photons eject electrons from their orbits by converting their energy into kinetic energy of freed electron; neutrons, on the other hand, interact with nuclei in a manner similar to photons. Depending on energy deposition patterns ionizing radiation is classified as either low- or high-LET. In both cases energy is deposited in the form of clusters (spurs) 1-4 nm in diameter, but with increase of LET a distance between spurs becomes smaller bringing them to a close proximity.

In terms of DNA damage, ionizing radiation acts through both direct and indirect mechanisms. The biological effects of X-rays, γ-rays or particles reflect either the direct absorption of radiation energy by DNA, or the formation of radicals in water and/or biomolecules, and their transformation into peroxyradicals in the presence of oxygen. The relative importance of direct and indirect effects of ionizing radiation on cytotoxicity and mutagenicity depends mostly on its quality and the conditions of radiation. In the case of sparsely ionizing radiation, indirect effects contribute to cell inactivation as much as 55%

in presence of oxygen, but only about 20% in its absence (von Sonntag, 1987). Double-strand and single-strand breaks, dsbs and ssbs (combined with other lesions leading to formation of dsbs) are considered the most deleterious lesions, directly correlating with cytotoxicity (reproductive cell death) and deletion formation (Ward *et al.*, 1985; Hall, 1988). They are formed in areas of high radical damage density and the ratio of dsbs to ssbs increases with LET (Kiefer, 1985).

1.3 Radiation Mutagenesis

Here we review accumulated data on the mutagenicity of ionizing radiation in the three most extensively studied genetic loci - *hprt*, *aprt* and *tk*. All three genes are nonessential and code for enzymes participating in purine (*hprt* and *aprt*) and pyrimidine (*tk*) salvage pathways. Protocols for the detection of forward mutations in these assays are essentially similar, being based on the inability of mutant cells to process hypoxanthine, guanine *(hprt)*, adenine (*aprt*) and thymidine (*tk*) in salvage pathways. Upon exposure to the respective toxic analogue, 8-azaguanine or 6-thioguanine (for *hprt*), 8-azaadenine or 2,6-diaminopurine (for *aprt*), and trifluorothymidine (for *tk*), wild type cells convert them to their nucleotides and incorporate them into DNA. This results in cell death after few rounds of replication. The principle difference between these assays is that *hprt* is a X-linked gene, whereas *aprt* and *tk* are autosomal genes present in genome in two copies, which permits recombination between homologous chromosomes.

1.3.1. Hypoxanthine Phosphoribosyltransferase

Hprt - has been a subject for extensive research during past 10 years, and has generally proven to be an informative endpoint for analysis of environmental mutagenesis

both *in vivo* and *in vitro* (see for review Cole *et al.*, 1990; Sankaranayranan, 1991; Robinson *et al.*, 1994; Cole and Skopek, 1994; Albertini *et al.*, 1993).

Data on the mutagenicity of ionizing radiation (Table 1) indicates that mutant induction rates are very similar in all cell types tested and ranges from 2 to 22 mutants per million viable cells per Gy. These values are relatively low compared to the mutagenicity of alkylating agents which may be more than 5-50-fold higher at equitoxic doses (Bradley *et al.*, 1988; Evans, 1994). The situation changes dramatically, however, when the *hprt* gene (or its bacterial analog *gpt*) is situated elsewhere in the genome. For example, the *gpt* gene autosomally integrated in CHO cells in a single copy exhibited about 10-fold higher mutagenic response than the endogenous *hprt* (Hsie *et al.*, 1990; Schwartz *et al.*, 1991). This suggests that there are other parameters involved which impose certain limitations preventing the recovery of some classes of mutants.

Spectrum of spontaneous mutations

The majority of mutations recovered at the *hprt* locus in T-cells from healthy unexposed adults and mammalian cell lines are point mutations. About 10-15% of *hprt* mutants have changes that can be detected in Southern blots (Cole and Skopek, 1994), whereas the fraction of gross rearrangements in lymphoblastoid cell lines may be considerably higher, constituting 29-63% of the mutants (Liber *et al.*, 1987; Gennet and Thilly, 1988; Tashibana *et al.*, 1990; Bao *et al.*, 1995). Mutants recovered from newborns also show an unusual prevalence of deletions in mutational spectrum (85%, Albertini *et*

Table 1. Mutagenicity of ionizing radiation at the *hprt* locus *in vitro*

Cell type	Treatment	Mutant induction rate (n x 10^{-6}/Gy)	Reference
TK6 +/-	X-rays	11	Amundson and Liber, 1991
TK6 -/+		12	
TK6	α-particles	25	Metting *et al.*, 1992
TK6	X-rays	8	Bao *et al.*, 1995
	radon	12	
L5178Y *tk* +/-	X-rays	12.5	Evans *et al.*, 1986
L5178Y *tk* +/0		8.5	
L5178Y *tk* +/-	X-rays	10	Evans *et al.*, 1990
CHO (log)	X-rays	10.2	O'Neill *et al.*, 1985
CHO	X-rays	2	Morgan *et al.*, 1990
CHO	X-rays	13.7	Fuscoe et al., 1992a
CHO D422 +/0	γ-rays	6.2	Breimer *et al.*, 1986
CHO D422 +/0	X-rays	1.7	Bradley *et al.*, 1988
D423 +/-		1,7	
T-cells	X-rays	10.6	Sanderson *et al.*, 1984
T-cells	X-rays	5.8	Sanderson and Morley, 1986
T-cells Go	γ-rays	8-9.5	O'Neill *et al.*, 1990
T-cells Go	X-rays	21.8	Imada and Norimura., 1994
Log		7.7	

Mutation induction rates were calculated from numerical data, or (when not available) from graphs at the doses with survival higher than 50%, assuming linear dose-response relationships.

al., 1990). These are specific to the loss of exons 2 and 3 (Lippert *et al.*, 1990). The analysis of deletion endpoints showed that these deletions occur in the regions containing the recognition sequence for V(D)J-recombinase (Fuscoe *et al.*, 1991), an enzyme involved in the process of T-lymphocyte maturation.

Spectrum of ionizing radiation- induced mutations

The spectrum of ionizing radiation-induced mutations is characterized by a sharp increase in the frequency of deletions (see Table 2). This is compared to the case of spontaneous events where the loss of entire *hprt* gene sequence is relatively uncommon, comprising between 0-29% in lymphoblastoid cells (Gennet and Thilly, 1988; Bao *et al.*, 1995; Tashibana *et al.*, 1990), 29% in CHO cells (Thacker and Ganesh, 1989), and 0-7% for human T-cells (Nicklas *et al.*, 1987; Hakoda *et al.*, 1989; Fuscoe *et al.*, 1992c).

Radiation-induced mutations exhibit a significantly higher percent of total deletions, up to 55% in *TK6* cells (Bao *et al.*, 1995), 43-69% in CHO cells (Morgan *et al.*, 1990: Fuscoe *et al.*, 1992a), 40-47% in human T-lymphocytes (Thacker, 1986; Nicklas *et al.*, 1991). At the same time, however, there are a few contradicting reports where the high proportion of total deletions was not observed (9%, T-cells - Skulimowski *et al.*, 1986; 0%, T-cells - O'Neill *et al.*, 1990). The size of deletions and the distribution of breakpoints along the sequence of *hprt* gene were analyzed in different studies (O'Neill *et al.*, 1990; Gennet and Thilly, 1988; Morgan *et al.*, 1990; Fuscoe *et al.*, 1992a, 1992b; Lippert *et al.*, 1995).

Table 2 Mutational specificity of ionizing radiation at the *hprt* locus *in vitro*

Cell type	Treatment	Changes in Southern Blots (%) spont.	induced	Reference
TK6	X-rays	36	54	Liber *et al.*, 1987
TK6	X-rays Radon	63	81 86	Bao *et al.*, 1995
TK6	γ-rays	29	47	Tashibana *et al.*, 1990
TK6	X-rays [131I]dUrd	57	51 62-84	Whaley and Little, 1990
CHO	γ-rays α-particles		70 73	Thacker, 1986
CHO	X-rays		81-93	Morgan *et al.*, 1990
CHO	X-rays		73	Fuscoe *et al.*, 1992a*
CHO D422 +/0	γ-rays		58.8	Breimer *et al.*, 1986
T-cells	X-rays		52	Skulimowski *et al.*, 1986
T-cells	γ-rays		43	Albertini *et al.*, 1989
T-cells	γ-rays		75	O'Neill *et al.*, 1990

(*) - deletion analysis by multiplex PCR method;

According to different reports, the breakpoints of induced deletions were clustered in the center of the gene (12 mutants - Morgan *et al.*, 1990) or distributed randomly (18 mutants - O'Neill *et al.*, 1990). A review of induced deletion mutants (Fuscoe *et al.*, 1992a) examines a total of 188 independent mutations and notes that in 68% of cases, both breakpoints were located within the gene, suggesting that they are not the result of two independent events. The size of recoverable deletions in *hprt* is basically determined by the distance between *hprt* target and the nearest essential gene. Fuscoe *et al.* (1992b) has shown that, among spontaneous mutants, deletions of at least 700 kb are tolerated at this locus. The maximum recoverable size of deletions was later extended to at least 3.5 Mb (Lippert *et al.*, 1995). It is thought that deletions greater than 1.3 Mb telomeric to *hprt* are not tolerated due to presence of putative essential gene between markers 342R and 592R (Nelson *et al.*, 1995).

Adenine Phosphoribosyltransferase

This enzyme is coded by an autosomal gene and maps to 16q24 in human (Chen *et al.*, 1991) and 3p in CHO cells. The *aprt* target is used in *in vitro* studies because of its autosomal location. This limits its use in *in vivo* studies as about 90% of the population is homozygous for *aprt*. For the purposes of *in vitro* studies several hetero- and hemizygous strains of parental CHO cells have been developed (Bradley and Letovanec, 1982; Thompson *et al.*, 1980). The two alleles in the heterozygous strains (*aprt* +/-) are characterized by readily detectable restriction fragment length polymorphism (RFLP), while *aprt* -/0 strains are hemizygous for not only the *aprt* locus, but also for flanking regions presumably harboring essential genes.

Experimental data accumulated to date suggest that mutagenicity of different agents at the *aprt* locus depends on specificity of mutagens, and the hetero- and hemizygotic status of strain employed (Table 3). For example, exposure of *aprt* +/- (AA8-5, D423) and *aprt* +/0 (AA8-16, D422) cells to the alkylating agent ethylmethane sulfonate (EMS) resulted in a strong and very similar in magnitude mutagenic response in both *aprt* and *hprt* regardless of zygosity (Bradley *et al.*, 1988). In contrast, the difference in X-rays-induced mutant frequencies at *aprt* locus was very large, at least 10-fold higher in heterozygous strains.

Table 3 Mutagenicity of ionizing radiation at the *aprt* locus

Cell line	Mutagen	Mutant induction		Reference
		aprt	*hprt*	
WR10 +/-	γ-rays	120		Fujimori *et al.*, 1992
CHO D422 +/0	γ-rays	0.7		Meuth, 1992
CHO D422 +/0	γ-rays	4.3	6.2	Breimer *et al.*, 1986
P19H22[c] +/-	γ-rays 252Cf	48.5 92		Turker *et al.*, 1997
CHO D422 +/0 CHO D423 +/-	X-rays	0.5 10.0	1.7 1.7	Bradley *et al.*, 1988

Spectra of Spontaneous Mutations

The spectra of spontaneous and induced mutations at the *aprt* locus are presented in Table 4. Generally, for the *aprt* locus in **hemizygous** strains intragenic deletions or chromosomal rearrangements are not prevalent. Most events involve point mutations (93% - Nalbantoglu *et al.*, 1983; 97% - Grosovsky *et al.*, 1986; 100% - de Long *et al.*, 1988; 81% - Klinedinst and Drinkwater, 1991; 92% - Meuth, 1992) The spontaneous

spectrum of point mutations was extensively analyzed in CHO D422 (+/-) cells, and was characterized by a high incidence of G:C to A:T transitions (73% - de Long *et al.*, 1988; 27% - Grosovsky *et al.*, 1988; 28% - Meuth, 1992). All of the deletions recovered spontaneously extended only into the 5'- flanking sequence (Grosovsky *et al.*, 1986; Nalbantoglu and Meuth, 1986), which is suggestive of presence of an essential gene located in the vicinity of *aprt* in the 3'-direction. In **heterozygotes,** the most frequent event was the loss of heterozygocity, LOH. Depending on cell type, LOH was observed in the majority (human fibroblasts 62% - Zhu *et al.*, 1993; human lymphoblasts - 85% - Fujimori *et al.*, 1992; 77%-Klinedinst and Drinkwater, 1991; mouse embryonal carcinoma cells - 95% - Turker *et al.*, 1995) or substantial minority of mutants (CHO D423 43% - Ward *et al.*, 1990). Based on estimates of the amount of genetic material in *aprt* fragments in Southern blots, the physical loss of the second allele was suspected in 19-59% of LOH mutants (Klinedinst and Drinkwater, 1991; Fujimory *et al.*, 1992).

Spectrum of Induced Mutations

In general, the induced mutational spectrum in *aprt* **heterozygotes** is dominated by the loss of the active allele. No significant increase in the initially high fraction of LOH mutants is seen and does not exceed 10% (Fujimori *et al.*, 1992; Turker *et al.*, 1995), with the physical loss of one allele in 10-100% of cases. The analysis of induced deletions in heterozygotes revealed that they might extend beyond 12 kb downstream of *aprt* (Bradley *et al.*, 1988).

In contrast, mutational spectra in *aprt* +/0 **hemizygotes** are dominated by point mutations. In a large collection of spontaneous (120) and induced (85) mutants derived from D422 cells, the visible alterations in SB were observed in only 22% of induced

mutants compared to 8% in spontaneous set. The remaining induced mutants carried all classes of base substitutions, small deletions and insertions, at the frequency not any different from spontaneous mutational spectrum (Meuth, 1992). In a similar study (Grosovsky *et al.*, 1986) changes in Southern blots were found in 16.4% (9 of 55) of γ-rays-induced mutants, compared to spontaneous 3%. About 70% of mutants with a normal Southern blot pattern had base substitutions, while the remaining 30% carried small deletions (Grosovsky *et al.*, 1988).

Thymidine Kinase

Thymidine kinase (*TK*) is one of the best-studied autosomal genes. *TK* +/- heterozygotes were developed from different mammalian cell lines including human (Liber and Thilly, 1982), hamster (Carver *et al.*, 1980) and mouse (Clive *et al.*, 1972) using a two-step protocol with frameshift mutagen ICR-191by selection of *tk* -/- mutants and then back selection of *tk* +/- revertants.

Initially, the mutagenic response at the *tk* locus was thought to approximate that of *hprt* (Liber and Thilly, 1982). Later, it was discovered that the mutants formed in this assay represent two classes, specifically a) colonies with the same doubling time as the wild type (NG); and b) slowly growing (SG) colonies (Yandell *et al.*, 1986). SG colonies frequently exhibit chromosome abnormalities associated with the *aprt* region (30% for L5178Y cells, Blazak *et al.*, 1989), and almost uniformly show loss of heterozygocity for *tk* locus and flanking sequences (*TK*6, Yandell *et al.*, 1990; Li *et al.*, 1992). With the addition of this class of mutants, radiation-induced mutant frequencies at the *tk* locus were found to be 1 to 3 orders of magnitude higher (depending on cell line) than at the

Table 4 Mutational specificity of ionizing radiation at the *aprt* locus

Cell type	Treatment	RFLP LOH (%)		Alterations in Southern blots (%)		Physical absence of allele		Reference
		SP	IND	SP	IND	SP	IND	
CHO D422 +/0	γ-rays			3	16.4			Grosovsky *et al.*, 1986
CHO D422 +/0	-			0				De Long *et al.*, 1988;
CHO D422 +/0	γ-rays			8	22			Meuth, 1992
CHO D422 +/0	γ-rays				24			Breimer *et al.*, 1986
CHO				6.7				Nalbantoglu *et al.*, 1983
CHO AA8-16 +/-	X-rays				100		100	Bradley *et al.*, 1988
LCL-721 +/-*	-	80				19.2		Klinedinst and Drinkwater, 1991
CHO D423 +/-	-	43						Ward *et al.*, 1990
H. Fibroblasts +/-	-	62						Zhu *et al.*, 1993
WR-10 +/-**	γ-rays	85	93			59	33	Fujimori *et al.*, 1992
P19H22***	γ-rays 252Cf	95 95	100			20	10	Turker *et al.*, 1995

(*)- human lymphoblastoid cell line; (**)- human lymphoblasts; (***) - mouse embryonal carcinoma cell line;
(SP) – spontaneous; (IND) – induced;

hemizygous *hprt* locus (Table 5). Interestingly, in *tk* +/0 cell line monosomic for chromosome carrying the *TK* gene, the induction kinetics is quite similar to that of *hprt* (Evans *et al.*, 1986), indicating that in hemizygous systems challenged with a mutagen inducing (presumably) gross alterations, a significant fraction of mutants of a certain class can not be detected.

Table 5 Mutagenicity of ionizing radiation at the *tk* locus

Cell line	Mutagen	Mutant induction ($n \times 10^{-6}$/ Gy)		Reference
		tk	***hprt***	
L5178Y-R16 *tk* +/- L5178Y-R83 *tk* +/0	X-rays	2100 20-40	12.5 8.5	Evans *et al.*, 1986
L5178Y-R16	X-rays	800	10	Evans *et al.*, 1990
L5178Y-R16	α-part	4800		Evans *et al.*, 1993
TK6 tk +/-**	Neutrons Argon	17.4 33.6		Kronenberg and Little, 1989
TK6 tk +/-	β- part.	45		Whaley and Little, 1990
TK6 +/- *TK6* -/+	X-rays	40 5	11 12	Amundson and Liber, 1991
TK6 +/-	α-part.	37.5	25	Metting *et al.*, 1992

(*) - L5178Y-R83 *tk* +/0 - strain monosomic for chromosome 11;
(**) - calculation has been made only for normally growing mutant colonies;

Spontaneous Mutational Spectra

Spontaneous mutants at the *tk* locus can be divided to three groups (Yandell *et al.*, 1990): 1) without any changes in Southern blot patterns; 2) with appearance of new bands on restriction maps, and 3) with the loss of allele polymorphism, but otherwise no changes in restriction pattern with any other enzyme. In a large group of spontaneous (171) *TK6*-mutants, rearrangements in Southern blots were observed in 3.5% of mutants, no changes

in SB with retained allelic polymorphism were seen in 8.2%, with the rest (88%) represented by the complete loss of the active allele. In a set of 36 spontaneous mutants (Grosovsky *et al.*, 1993), LOH accounted for 58% of mutants, while 11% were due to structural rearrangements. Of the mutants with point mutations, 75% carried base substitutions. In another test system with the mouse lymphoma cell line, L5178Y, 96% of mutants showed a loss of heterozygocity (LOH) event (Evans *et al.*, 1990).

Induced Mutational Spectra

Ionizing radiation does not produce noticeable shifts in mutational spectrum at the *tk* locus. With human *tk* heterozygotes, the fraction of mutants with LOH ranged from 47 to 65% (Amundson and Liber, 1991; Yandell *et al.*, 1990; Kronenberg and Little, 1989). In irradiated L5178Y cultures, the percentage of LOH mutants was very high (95%, Evans *et al.*, 1990). In a small sample (4) of LOH mutants analyzed by Evans *et al.* (1993) using Southern blots the density of bands containing *tk* was the same as in WT samples, indicating that both alleles were physically present. The fraction of mutants with intragenic rearrangement ranged from 0 to 21.3 % (Yandell *et al.*, 1990; Kronenberg and Little, 1989). The scale of events leading to LOH in irradiated cultures was evaluated using analysis of polymorphic markers on chromosome 17 c-erbA1 (17q11-q12.21, Xu *et al.*, 1988) and D17S2 in *TK6* cells. In spontaneous mutants loss of c-erbA1 allele accompanied *TK*(-) phenotype in 33% of NG colonies and 51% of SG mutants (Yandell *et al.*, 1990). In NG fraction of radiation-induced mutants with concomitant loss of 17 c-erbA1 constituted 30%, which is not different from spontaneous values. Unfortunately, SG radiation-induced mutants were not analyzed in this study and the data may not be

fully representative of induced mutational spectrum, because 80% of the induced mutants fall in this category (Amundson and Liber, 1991).

Table 6 Mutational specificity of ionizing radiation at the *TK* locus

Cell type	Mutagen	RFLP LOH (%) SP	RFLP LOH (%) IND	Alterations in Southern blots (%) SP	Alterations in Southern blots (%) IND	Reference
L5178Y-R16	X-rays	96	95			Evans *et al.*, 1990
L5178Y-R16	γ-rays				0	Evans *et al.*, 1993
TK6	Neutrons Argon		65 60		11.5 21.3	Kronenberg and Little, 1989
TK6	X-rays	88	60*	3.5	0	Yandell *et al.*, 1990
TK6 +/- *TK6* -/+	X-rays		 47**			Amundson and Liber, 1991
TK6	X-rays	58		11		Grosovsky *et al.*, 1993

(*) - spontaneous mutants - both NG and SG colonies, X-ray-induced - NG clones only;
(**)- percentage of both LOH and rearrangements:

1.4 Radiation in Space

Space presents an entirely new environment for humans, and possesses several unique qualities. Radiation in space comes from three major sources including galactic cosmic radiation, solar radiation, and trapped particles radiation complemented by the secondary emissions caused by collisions of charged particles with the shielding material.

The major components of galactic cosmic radiation (GCR) are high-energy protons and heavier ions (HZE) with mostly even atomic numbers (about 98%, Simpson, 1983). The majority of these particles are comprised of hydrogen, helium, carbon and iron, which are present isotropically in space and come from sources outside our solar system. The spectrum and intensity of GCR can be modulated by solar events ("solar wind")

causing an increase of the interplanetary magnetic field, which in turn deflects a large portion of GC rays, especially its lower energy fraction (NCRP, 1989). As a result, the intensity of GCR may fall as much as 10 times during solar events (Radiation Hazards to Crews on Interplanetary Missions, 1996).

Solar radiation is mostly composed of protons, with a small contribution of helium ions, HZE particles, and electrons. The largest solar particle events usually occur during the active periods of the solar cycle; they may last several days, and have fluence of more than 10^{10} protons cm^{-2} with energies greater than 10 MeV (Vahia and Biswas, 1983). In addition, anomalously large solar particle events have been described (King, 1974), which may increase the intensity of solar radiation by several orders.

A considerable volume of space surrounding the Earth is occupied by particles (electrons and protons) trapped in the Earth's magnetic field. The particles spiral along the geomagnetic field lines, and are spatially attributed to so called "outer" (2.8-10 earth radii, Re) and "inner" (<2.8 Re) zones. Electron intensities in the outer zone are about 10 times greater (Stauber *et al.*, 1983), and the highest density of fluence is registered at the distance of 4.5Re (Problems of Space Biology, 1989). Protons occupy more limited volume and are more intense in the region of the South Atlantic Anomaly. The intensity of trapped particle radiation is also very sensitive to solar activity and magnetic storms, which can cause large fluctuations over a short period of time.

About one third of the radiation affecting astronauts is delivered in the form of high-LET radiation (Fry, 1992). It has been deduced that during 6 months in space, a sample of 1000 cells would have been hit by 12 particles with LET > 10 KeV/μm, and 0.5 particle with LET > 50 KeV/μm. Doses and dose-rates of exposure are very low and

range from 0.01 to 1.0 mGy per day (NCRP, 1989). However, the fact that a substantial portion of exposure consists of high-LET radiation (Brenner, 1992) creates a higher risk for astronauts due to some fundamental differences in the biological effect.

1.4.1 Relative Biological Efficiency of High LET Radiation.

There is a large body of evidence (Table 7) indicating that relative biological effectiveness (RBE) of high-LET radiation is considerably higher than that of conventional radiation. A number of studies have shown that depending upon the selected end-point and composition, the RBE of high-LET radiation may be up to 60-fold that of conventional radiation (see Table 7).

Regardless of quality, ionizing radiation produces single-strand breaks, double-strand breaks and base modifications each of which may contribute to induced mutagenesis (Von Sonntag, 1987). It should be noted, however, that modified bases resulting in point mutations make only a minor contribution to either low- and high-LET mutational spectra (Ward, 1995), and are detectable only in assays with low tolerance to deletions (Liber *et al.*, 1986; Takimoto *et al.*, 1993; Yuan *et al.*, 1995). Mutational spectra derived from cells exposed to low-LET or high-LET radiation are essentially similar, and exhibit a sharp increase in the incidence of deletions (Whaley and Little, 1990; Schwartz *et al.*, 1991; Nicklas *et al.*, 1990). However, in high-LET induced spectra the occurrence of large-scale rearrangement is complemented with increased incidence of intragenic deletions not observed after X-ray exposure (Kronenberg and Little, 1989). These subtle differences in mutational spectra are mirrored in recent findings indicating that, unlike

Table 7 Relative Biological Effectiveness of high-LET radiation.

Cell type	Radiation type	End-point	RBE	Reference
CHO-K1 CHO-10T5	α-particles (212Bi) Fission-spectrum neutrons	*hprt* gpt *tk*	4-6 4-6 >12	Schwartz et al, 1991
Primary human fibroblasts	Helium-3 ions 10 KeV/μk 150 KeV/μk	Cytotoxicity *hprt*	1.3 9.4	Hei *et al.*, 1988
Human T-cells	fission neutron, 1.6 MeV	micronuclei	12.2	Huber *et al.*, 1994
Aspergillus nidulaus	β-particles α-particles	cytotoxicity	2.1 3.4	Normansell and Holt, 1979
C3H10T1/2	α-particles 33 KeV/μk deuterons 18 KeV/μk	cytotoxicity	4.2 2.2	Bettega *et al.*, 1998
C3H10T1/2	α-particles <120 KeV/μk	transformation	20	Miller *et al.*, 1995
C3H10T1/2	α-particles	transformation	10	Hieber *et al.*, 1987
C3H10T1/2	Neutrons 5.9 MeV 0.35 MeV	transformation	13 35	Miller *et al.*, 1990
Human T-cells	Nitrogen ions	apoptosis	3	Meijer, 1998
Human fibroblasts	Neutrons 0.22-13.6 MeV	aberrations	24.3	Pandita and Geard, 1996
Human epithelial H184B5 F5-1 M/10	α-particle 109 KeV/μk	cytotoxicity aberrations	3.3 5	Durante *et al.*, 1995
Syrian Hamster Embryo cells	α-particles 90-200 KeV/μk	cytotoxicity transformation	3.6-7 60	Martin *et al.*, 1995
C3H10T1/2	α-particles 177 KeV/μk	cytotoxicity	5.2	Napolitano *et al.*, 1992

conventional radiation, high-LET exposure produces non-random distribution of DSBs, which is in agreement with the concept of multiply damaged sites put forward by Ward *et al.* (1985). The analysis of distribution of fragment size upon exposure to low- and high-LET radiation reveals that the latter induces an excess of fragments smaller than 1 kb (Newman *et al.*, 1997; Lobrich *et al.*, 1996; Rydberg, 1996) which may comprise 20-90% of total number of DSBs (Rydberg, 1996). At the same time the induction rate for widely spaced DSBs is diminished (Lobrich *et al.*, 1996). These finding are in agreement with the model proposed by Holley and Chatterjee (1996) viewing a 30 nm periodically structured chromatin fiber as a target for particles. According to the model, high-LET radiation creates local (=<40 bp) and regional (kb) clustering of DNA lesions reflecting symmetries in a fiber structure and yielding short fragments of uniform length.

The higher complexity of high-LET-induced DNA lesions eliminates the advantage of having a template for their repair (Ward, 1985). Although the RBE for breakage is close to unity for high-LET, the rates of repair decrease with increase of LET (Ritter *et al.*, 1977; Hendry, 1991; Goodwin *et al.*, 1994; Heilmann *et al.*, 1993; Frankeburg-Schwager *et al.*, 1994). This probably explains the very high RBE of high-LET radiation for cytotoxicity.

1.4.2 Protracted/Fractionated Exposure *vs.* Acute Exposure

Fractionated and/or protracted *versus* acute exposure to the same cumulative dose is another interesting aspect of biological consequences of high-LET radiation. A significantly higher RBE of protracted exposure for oncogenic transformation was first noted by Hill *et al.* (1982). This is relevant to the risk assessment for astronauts because

on their normal, low orbit they are subjected to fractionated exposure of fast protons (50-350 MeV), trapped by the Earth's magnetic field in South Atlantic Anomaly (NCRP, 1989; Nguyen *et al.*, 1990). This phenomenon is termed "inverse dose-rate effect" (Rossi and Kellerer, 1986), and was observed in several experiments employing oncogenic transformation in mouse embryo cells C3H10T1/2 and other cell lines (Hill *et al.*, 1984; 1985; Miller *et al.*, 1990; Brenner and Hall, 1990). The model developed by Rossi and Kellerer (1986) postulates that the higher RBE of protracted exposure to ionizing radiation is due to 1) a larger number of cells affected in a radiosensitive stage of the cell cycle, and 2) relatively higher energy deposition by high-LET particles which even at low doses can cause DNA damage. For both low- and high-LET radiation, the most sensitive phase of the cell cycle appears to be G1 phase (Chuang *et al.*, 1996; Leonhardt *et al.*, 1997).

However, not all experiments with prolonged exposure yield similar results (Hieber *et al.*, 1987; Di Majo *et al.*, 1994; Saran *et al.*, 1994) suggesting a somewhat more complex mechanism. The model by Brenner and Hall (1990) links intervals between exposures to the duration of radio-sensitive period in the cell cycle, as well as the fractionated dose with the LET value, and eventually the dose to the nucleus. This model allows the quantitative prediction of the potential enhancement of RBE upon the fractionation for radiation of different qualities. Interestingly, according to this model, the inverse dose-rate effect should not be seen for HZE particles, but is expected in the case of trapped protons.

It should be noted that for the expression of the inverse dose-rate effect, energy deposition has not only a lower but also an upper limit (Brenner *et al.*, 1993) possibly due

to the factors not considered in the above model. A large data set derived from experiments using essentially similar approaches indicate that application of a small dose prior to a much larger dose of radiation may lead to opposite results, inducing considerably lower biological effect than after the large exposure alone (Sanderson and Morley, 1986; Sankaranarayanan *et al.*, 1989; Kelsey *et al.*, 1991; Wolff *et al.*, 1991; Bai and Chen, 1993; Joiner *et al.*, 1996). This effect of the small priming dose is termed "the adaptive response", which may be attributed to induction of repair mechanisms that allow cells to cope with larger dose with higher efficiency. Apparently, every particle of a certain LET has its own dose threshold for the induction of protective mechanisms. In this context, the inverse dose-rate effect may be induced as long as the energy deposited is sufficient to cause DNA damage, but not sufficient to sound the alarm and induce the adaptive response.

1.4.3 Confounding Factors and RBE in Space

Apart from radiation, there are several other factors aboard spacecraft that may interfere with cellular reactions. Of these, a major component may be microgravity. According to some reports, microgravity seriously impairs proliferation of the cells (Cogoli *et al.*, 1984; Meehan, 1987), possibly via downregulation of expression of involved genes (de Groot *et al.*, 1990). It should be noted, however, that DNA synthesis and mitogen activation tests on lymphocytes from rats flown on Cosmos 2044 mission did not show any difference from ground control (Nash *et al.*, 1992).

Also, the spacecraft environment is not free from airborne mutagens/carcinogens. Analysis of aboard atmospheric samples revealed presence of several carcinogens such as acetaldehyde, dichloromethane, formaldehyde, isoprene, 1,2-dichloroethane, acrolein, benzene and furan in measurable concentrations (James, 1997).

Recent cytogenetic studies performed on lymphocytes taken from MIR-18 crews (flights duration 6 months) revealed that in terms of chromosomal aberrations, the effectiveness of space radiation ranges from 2.8 to 3.5 (Yang *et al.*, 1997a; 1997b). In these studies post-flight samples showed a considerable excess of chromosomal aberrations, but not sister chromatid exchanges. Similar results were obtained by Testard *et al.* (1996) in cosmonaut blood samples after a 6-month period in space.

1.5 Discussion

Available data on radiation mutagenesis in *hprt*, *aprt* and *tk* genes indicate that the mutagenic response in autosomal genes is qualitatively and quantitatively different from that of hemizygous targets. Mutation induction rate in heterozygotes may be several-fold higher, indicating that majority of mutations is not recoverable in *HPRT* assay or *aprt* hemizygous strains.

The nature of non-recoverable mutations in hemizygotes may be inferred from data on changes in induced mutational spectrum, indicating that exposure to ionizing radiation results in a higher fraction of deletions. Comparison of mutant induction and mutational spectra in *hprt* and aprt +/0 strains suggests that in case of mutagens producing large-scale rearrangements, the recoverability of mutants is restricted by the distance between the selectable locus and the nearest essential gene. In this regard *hprt* as a selectable locus

allows recovery of deletions spanning up to 3.7 Mb (Lippert *et al.*, 1995). Compared to *hprt*, the hemizygous *aprt* system is less flexible, due to its inability to recover deletions extending in the 5-prime direction because of presumptive upstream essential gene (Breimer *et al.*, 1986). This is reflected in somewhat lower mutant induction (Breimer *et al.*, 1986; Bradley *et al.*, 1988) and smaller fraction of deletions in mutational spectrum in *aprt* spontaneous and induced mutants (Grosovsky *et al.*, 1986; Meuth, 1992). By the same token, rearrangements induced by ionizing radiation (and other potent clastogens) are likely much larger, because in heterozygotes systems recovery of mutants is several-fold higher than in *hprt* (DeMarini *et al.*, 1987; Moore *et al.*, 1987; sections 1.3.2. and 1.3.3).

At the same time all three markers provide a reliable measurements of mutagenicity induced by point mutagens. In several studies with simultaneous determination of mutagenic response, *hprt*, *aprt* and *tk* loci showed very similar reactions. For example, the mutagenicity of EMS in CHO cells (Thompson *et al.*, 1980; Bradley *et al.*, 1988) was virtually identical for *hprt* and *aprt* in all strains. Similar results were obtained for *hprt* and *aprt* when mutant frequencies were measured in CHO cells after exposure to ICR-191 and UV (Thompson *et al.*, 1980).

For heterozygotes the most common change in spontaneous and induced mutants is the loss of one allele (see sections 1.3.2. and 1.3.3). The scale of LOH in spontaneous and induced mutants can be assessed via the determination of the presence of polymorphic markers on the chromosome. For induced *aprt* mutants, LOH extending into adjacent markers is actually less characteristic than for spontaneous mutants (Fujimori *et al.*, 1992), and the proportion of mutants with LOH restricted exclusively to selectable locus is higher

(Turker *et al.*, 1995). The same phenomenon is observed in the *tk* gene where the extent of lesions inactivating distant markers in spontaneous and induced LOH mutants is either very similar (Yandell *et al.*, 1990), or considerably more localized for induced groups (Evans, 1994; Li *et al.*, 1992). The phenomenon of LOH is structurally complex and may reflect any of the number of mechanisms including nondisjunction, deletions, recombination or gene conversion. There is little information available on the contribution of each of these mechanisms to the expression of the recessive phenotype. Karyotyping and analysis of LOH in distant markers have shown that nondisjunction is not involved in any LOH, spontaneous or induced, *tk* mutant (Yandell *et al.*, 1990; Li *et al.*, 1992). Densitometric analysis gave contradicting results regarding incidence of simple deletions in LOH mutants. According to different reports, the fraction of deletions ranged from 20 to 59% in spontaneous, and from 10 to 33% in induced *aprt* LOH mutants (Bradley *et al.*, 1988; Fujimory *et al.*, 1992; Turker *et al.*, 1995), *i.e.* actually decreased. In contrast, in a study carried out by Li *et al.* (1992) most of the induced *tk* LOH appeared to arise from simple deletion events, whereas recombination likely accounted for the majority of spontaneous mutants. Considering the permissive nature of diploid genes towards deletions, it is likely that their representation in both spontaneous and induced spectra should be somewhat higher than in *hprt*. This is actually not the case (Tables 4 and 6), which may be viewed as a circumstantial evidence against the prevalence of deletions in LOH phenotype in induced mutants. There is also some evidence that exposure to mutagens generally increases recombination via DNA repair and transcriptional activity (Hellgren, 1992). It is shown, for example, that transcription at activation is accompanied by an increase in the frequency of recombination (Schlissel and Baltimore, 1989), and that

exposure to mutagens activates transcription of many genes, including *aprt* and *tk* (Kleinberger *et al.*, 1988; Benjamin and Little, 1992).

From the available data it may be inferred that somatic recombination and (less probably) deletions are the major mechanisms for ionizing radiation-induced conversion to a recessive phenotype in diploid genes. On the other hand, there is reason to believe that somatic recombination plays a very important role in preventing cell accumulation of mutations. In this regard, the CHO hybrid cell line AL-J1 carrying the stably integrated human chromosome 11 (Waldren *et al.*, 1986) offers a unique opportunity to analyze mutagenesis in genes, which are located on a non-essential chromosome (*i.e.* recoverable size of lesion is not restricted), and incapable of recombination due to the absence of an homologous counterpart. In AL-J1 cells the mutagenic response of marker genes after exposure to ionizing radiation was more than 200-fold higher than values obtained using conventional methods (Waldren *et al.*, 1986). This indicates that mutant induction in somatic genes, although substantially higher than in hemizygous genes *in vivo* (*i.e.* HLA-A - Janatipour *et al.*, 1988; Morley *et al.*, 1990), could have been extremely high without recombination. From the results of this experiment, along with the fact that i) ionizing radiation is a relatively weak mutagen, and ii) the fraction of deletions recovered in heterozygotes is not higher than in hemizygotes, it may be assumed that in somatic genes another strategy of dealing with DNA lesions is employed. LOH as a major type of reaction to large-scale DNA damage may provide a mechanism of nonspecific selection against large deletions. In this case, there are three possible scenarios:

1) The LOH in favor of a defective allele - which results in nonviable cell not contributing to accumulation of mutations (nonviability is caused by disappearance of both alleles of essential gene along with selectable marker);

2) The LOH in favor of the WT allele - which results in restoration of diploid WT genotype and renders a perfectly normal cell;

3) Retention of cell viability in absence of mitotic recombination, but through gene dosage effect its clonal expansion will be hampered and this clone will thus be outgrown and eventually diluted from the organism;

The hemizygous nature of the *hprt* gene and therefore the ease with which mutants can be isolated and analyzed makes *HPRT* assay a very convenient tool for the monitoring of the genetic consequences of exposure to environmental mutagens in human populations *in vivo*, as well as for the *in vitro* studies. At the same time it should be kept in mind that this genetic endpoint has serious limitations, which must be considered in the process of interpretation of *HPRT* data. These limitations also result from its hemizygosity and include:

1) The relatively poor recoverability of mutants with large scale rearrangements, and 2) Difference in the mechanisms of mutagenesis in X-linked and somatic genes.

From the reviewed data, it may be concluded that mutation accumulation rates in different regions of the genome are not uniform. Indeed, they may vary by 2-3 orders in magnitude. Hence, accurate risk estimates can not be based solely on *hprt* data and must be complemented by the data derived from other assays employing somatic genes. The currently available assortment of diploid genetic endpoints for *in vitro* studies includes artificially constructed heterozygous cell lines with one allele inactivated. This is

substantially different from an *in vivo* situation, and results on mutability obtained from these assays therefore may also not be fully representative of *in vivo* mutagenic responses in diploid loci. It is thus necessary to develop new *in vitro* assays employing somatic genes and allowing detection and molecular analysis of mutants in mammalian cells where both alleles are initially active.

2. *Hprt* as a Genetic Target

2.1 Background

Hprt is housekeeping, non-essential gene located in the X chromosome at the position Xq26-27 (Pai *et al.*, 1980), hemizygous in males, and functionally hemizygous in females due to the inactivation of the second X-chromosome. It belongs to the group of "salvage" proteins, and is responsible for recycling of up to 90% of free purines in human cells (Lehninger, 1978) as opposed to energetically much costlier *de novo* synthesis. It is expressed in all somatic tissues although level of expression significantly varies, being several-fold higher in brain (Melton *et al.*, 1981). RNA expression and cellular concentration of the enzyme are also cell cycle dependent and rise sharply in dividing cells (Steen *et al.*, 1990).

The normal enzyme exists in a dimeric or tetrameric form (Holden and Kelley, 1978; Johnson *et al.*, 1979) each monomer containing 217 amino acids (Wilson *et al.*, 1982). It catalyses a Mg2+-dependent reaction of condensation of bases hypoxanthine and guanine with 5'-phosphoribosyl-1-pyrophosphate (PRPP) resulting in a transfer of ribosyl phosphate group to N9 nitrogen of the bases, and the formation of 5'-IMP and 5'-GMP

respectively (Fig. 1). In addition, the enzyme recognizes other purine analogs, *e.g.*, 6-mercaptopurine, 6-thioguanine, and 8-azaguanine (Klenitsky *et al.*, 1970), which when present result in the killing of cells with a functional (wild type) *hprt* gene after few rounds of replication (Wotring *et al.*, 1980). Actual or functional hemizygocity of *hprt* along with guanine analogs cytotoxic specifically for wild type cells a served as a basis for development of *hprt* mutation assay (Albertini *et al.*, 1982), which gained a considerable popularity over the last 15 years.

6-Thioguanine (6TG) is the most extensively (almost exclusively) used in *hprt* assay toxic analog of guanine. The exact mechanism of its action is not yet completely elucidated. 6TG is recognized by *hprt* as a substrate and is incorporated into tRNA (Morgan *et al.*, 1994) and DNA (Nelson *et al.*, 1975), producing mispairing (Griffin *et al.*, 1994) and DNA strand breaks (Christie *et al.*, 1984). The incorporation of the toxic analog appears to be a major cause of cytotoxicity, because the inhibition of DNA synthesis by arabinosylcytosine protects cells from 6TG action (Nelson *et al.*, 1975). In addition, 6TG exhibits a delayed cytotoxic effect arresting cells in the second G2 phase (Worting *et al.*, 1980). Recently it has been found that incorporated 6TG is methylated *in vivo*, and the resulting S6-methylthioguanine mispairs with T (Swann *et al.*, 1996). The mismatch is recognized by the hMutS alpha mismatch-binding complex. The strength of binding depends on the preceding base (G > C = A > T) (Waters and Swann, 1997). Post-replicative repair provokes chromosome aberrations, and is hypothesized to be responsible for delayed cytotoxicity of 6TG (Swann *et al.*, 1996).

2.2 Structure of the *Hprt* Gene

The genomic sequence of *hprt* spans almost 57 kilobases (Edwards *et al.*, 1990), and is composed of 9 exons separated by non-coding sequences of different length, ranging from less than 200 b to more than 13 kb. A 400-nucleotide sequence upstream from the initiation codon contains 75% of G and C residues, with 6 hexanucleotide runs (5'-GGCGGG), which are positioned within two 27 bp repeated sequences known as the bi-directional SV40 promoter binding transcription factor Sp1 (Kim *et al.*, 1986). Typically for mammalian housekeeping genes this sequence does not contain TATA or CAAT boxes (Kim *et al.*, 1986; Patel *et al.*, 1986). Processed RNA yields a 1.6 kb molecule carrying 654 bp of coding sequence (Jolly *et al.*, 1983).

2.3 *Hprt* as a Target for Mutational Analysis

Beside several advantages of *hprt* as an endpoint for mutagenesis and mutagenicity studies mentioned above, it also has certain shortcomings. One of the well documented phenomena of *hprt* assay is the inverse relationships between mutant frequencies (MF) and plating efficiencies (PE), that was reported independently by number of groups (Cole *et al.*, 1988, 1991; Davies *et al.*, 1992; Branda *et al.*, 1993). These observations indicate a significant recovery bias, although its direction is not yet unambiguously determined. As a target, *hprt* has rather limited ability of detecting genomic rearrangements exceeding 3.5 Mb in size (Lippert *et al.*, 1995). Also, as a method based on the phenotypic selection of mutations (and hence on tissue culture), the *hprt* assay does not permit detection of mutants with high residual enzyme activity (Table 8), or in all tissues of potential interest.

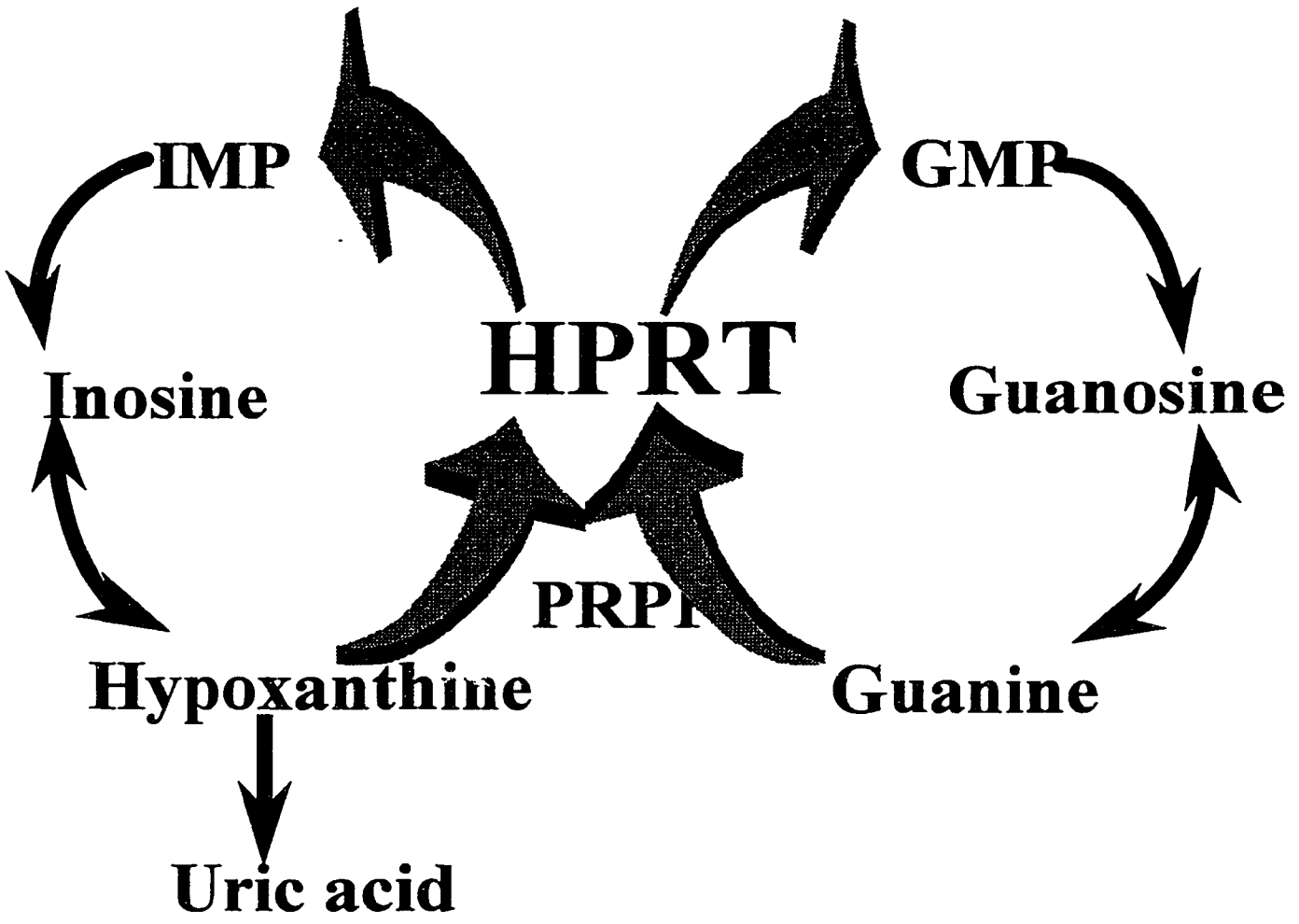

Figure 1. Hprt function
Salvage pathway enzyme which catalyses the condensation of 5'-phosphoribosyl-1-pyrophosphate (PRPP) and the purine bases hypoxanthine and guanine to form 5'-inosine monophosphate and 5'-guanisine monophosphate.

2.3.1 Recoverability of Mutations and Effective Target Size

There are at least two ways of creating an *hprt* negative phenotype. The first may be caused by a mutation outside the coding sequence that leads to a significant reduction of gene expression. The second mechanism involves mutation within the coding sequence or in the splice sites. Several reports indicate that *hprt* m-RNA expression in mutant cells is commonly lower that in WT cells (*e.g.* Steen *et al.*, 1990). Similarly, data on enzyme activity in mutant cells selected in *hprt* assay reveals that *hprt* activity in more than 90% of mutants is less than 10% (Riddle and Hsie, 1978; Alvi and Williams, 1992; Steen *et al.*, 1993; Table 8).

Table 8. Residual *HPRT* activity in mutant clones

Hprt Activity % of control	Riddle and Hsie (1978)	Alvi and Williams (1992)	Steen *et al.* (1993)	TOTAL	(%) of total
0-2	9	7	13	29	46.0
2-5	3	11	3	17	27.0
6-10	1	10	0	11	17.4
11-15	2	0	1	3	4.8
>16	0	2	1	3	4.8
				63	**100.0**

This indicates that the requirement for phenotypic selection with 6TG does not permit the detection of mutants with intermediate phenotype. Therefore, it can be assumed that mutations should tend to concentrate within functional domains of coding sequence. For example, a study performed by Lambert and co-workers (1992) on the datasets from normal individuals, Lesch-Nyhan and gout patients revealed that distribution of mutations along the *hprt* coding sequence was far from random and showed clustering within certain relatively short regions. Alignment of the mutation distribution pattern with homologous

regions in *hprt* cDNA derived from human and taxonomically distant species (*Schistosoma mansoni* and *Plasmodium falciparum*) showed that 76% of all missense mutations fell within evolutionary conserved sequences (Lambert *et al.*, 1992).

In Figure 2, we compare human *HPRT* protein sequence with enzyme sequences from several mammalian species as well as from Salmonella and Plasmodium (Lee *et al.*, 1998; Konecki *et al.*, 1982; Rossiter *et al.*, 1991; Vasanthakumar *et al.*, 1989; Jansen *et al.*, 1992), which share about 22.5% (49/218) identity. In addition, in a number of positions, amino acids are identical in 6 out of 7 sequences, whereas a 7^{th} sequence carries a conservative substitution (by a residue with similar properties). These highly conserved amino acids comprise 25.7% of the protein sequence. Conserved positions generally correspond to amino acids involved in formation of enzyme active center and dimer interface (Eads *et al.*, 1994). However, there also are several conserved codons with no known assigned function.

Hprt database has considerably expanded over the last few years (Cariello, 1996) and presently contains 753 spontaneous and induced base substitutions, of which 628 are mis-sense mutations. Analysis of distribution of mis-sense mutation in the *hprt* coding sequence (Fig. 3) shows obvious clustering within relatively short conserved motifs, which is similar to findings reported by Lambert *et al.* (1992). Accordingly, compared to the rest of the sequence, conserved regions harbor the major fraction of mutations (72.3%, Fig.4a, b). Average number of mutations per codon in non-conserved group (denoted as NC in graphs) is 1.54 ± 0.26, whereas in highly conserved residues it increases to 3.16 ± 0.64 (two tail $p = 0.022$). This value is even higher (5.65 ± 1.10; $p=0.0007$) in identical regions. It should be noted however, that several conserved positions exhibit a very low incidence

of mutations in the *Hprt* database. This can be probably explained by presumably higher residual enzyme activity resulting from changes in these codons, which precludes recovery of mutations under the stringent conditions of the *hprt* assay (see Table 8). This explanation is supported by the distribution of mutations rendering either partial (gout), or severe (Lesch-Nyhan, LN) deficiency (Fig. 3), showing that the majority of LN mutations are found in positions with high yield of mutations, whereas several mutations from gout patient are located in 'silent' codons.

As can be assumed from available data, saturation of *hprt* sequence with mutations is most likely biphasic, and exhibits high rate (high saturation) for conserved sequences, and low rate (low saturation) for non-conserved sequences. Taken together these data indicate that effective size of *hprt* as a target for mutagenesis is restricted to conserved regions which include approximately 105 codons dispersed within the coding sequence.

```
                          5                  20                  35                  50                  63                  78
1 Salmonella ---------------- ---------------- --------MKHTVEV MIPEAEIKARIAELG RQITERYKDSG-SEMV LVGLLRGSFMFMADL
2 Homo       ----------MATRS PGVVISDDEPGYDLD LFCIPNHYAEDLERV FIPHGLIMDRTERLA RDVM---KEMGGHHIV ALCVLKGGYKFFADL
3 Rattus     ----------MSTLS PSVVISDDEPGYDLD LFCIPNHYAEDLEKV FIPHGLIMDRTERLA RDVM---KEMGGHHIV ALCVLKGGYKFFADL
4 Mus        ----------MPTRS PSVVISDDEPGYDLD LFCIPNHYAEDLEKV FIPHGLIMDRTERLA RDVM---KEMGGHHIV ALCVLKGGYKFFADL
5 Cricetulus ----------MATRS PSVVISDDEPGYDLD LFCIPNHYVEDLEKV FIPHGVIMDRTERLA RDVM---KEMGGHHIV ALCVLKGGYKFFADL
6 Meriones   ----------MATRS PSIVIGDDEPGYDLD LFCIPKHYAEDLEKV FIPHGLIMDRTERLA RDVM---KEMGGHHIV ALCVLKGGYKFFADL
7 Plasmodium MPIPNNPGAGENAFD PVFVNDDD--GYDLD SFMIPAHYKKYLTKV LVPNGVIKNRIEKLA YDIK---KVYNNEEFH ILCLLKGSRGFFTAL

                         91                 105                 119                 134                 149                 164
1 Salmonella CREVQ---------- -VPHEVD-FMTASSY GSGMSTTRDVKILKD LD-EDIRGKDVLIVE DIIDSGNTLSKVREI LGLREPKSLAICTLL
2 Homo       LDYIKALNRNS--DR SIPMTVD-FIRLKSY CNDQSTG-DIKVIGG DDLSTLTGKNVLIVE DIIDTGKTMQTLLSL VRQYNPKMVKVASLL
3 Rattus     LDYIKALNRNS--DR SIPMTVD-FIRLKSY CNDQSTG-DIKVIGG DDLSTLTGKNVLIVE DIIDTGKTMQTLLSL VKQYSPKMVKVASLL
4 Mus        LDYIKALNRNS--DR SIPMTVD-FIRLKSY CNDQSTG-DIKVIGG DDLSTLTGKNVLIVE DIIDTGKTMQTLLSL VKQYSPKMVKVASLL
5 Cricetulus LDYIKALNRNS--DR SIPMTVD-FIRLKSY CNDQSTG-DIKVIGG DDLSTLTGKNVLIVE GIIDTGKTMQTLLSL VKRYNPKMVKVASLL
6 Meriones   LDYIKSLNRNT--DR SIPMTVD-FIRLKSY CNDQSTG-DIKVIGG DDLSALTGKNVLIVE DIIDTGKTMQTLLSL VQKYSPKMVKVASLL
7 Plasmodium LKHLSRIHNYSAVET SKPLFGEHYVRVKSY CNDQSTG-TLEIVS- EDLSCLKGKHVLIVE DIIDTGKTLVKFCEY LKKFEIKTVAIACLF

                        179                 194                 206         218
1 Salmonella DKPSRREVDVPVEFV GFSIPDEFVVGYGID YAQRYRHLPYVGKVV LLDE----------     178
2 Homo       VKRTPRSVGYKPDFV GFEIPDKFVVGYALD YNEYFRDLNH---VC VISETGKAKYKA---     218
3 Rattus     VKRTSRSVGYRPDFV GFEIPDKFVVGYALD YNEHFRDLNH---VC VISESGKAKYKA---     218
4 Mus        VKRTSRSVGYRPDFV GFEIPDKFVVGYALD YNEYFRNLNH---VC VISETGKAKYKA---     218
5 Cricetulus VKRTSRSVGYRPDFV GFEIPDKFVVGYALD YNEYFRDLNH---IC VISETGKAKYKA---     218
6 Meriones   VKRTPRSVGYRPDFV GFEIPDKFVVGYALD YNEYFRDLNH---VC VISESGKAKYKA---     218
7 Plasmodium IKRTPLWNGFKADFV GFSIPDHFVVGYSLD YNEIFRDLDH---CC LVNDEGKKKYKATSL     231
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Figure 2. Conserved aminoacids in the *hprt* gene. Red - All residues identical (ID); Green - highly conserved (6 identical + 1 conservative change (HC); Black - non conserved (NC);

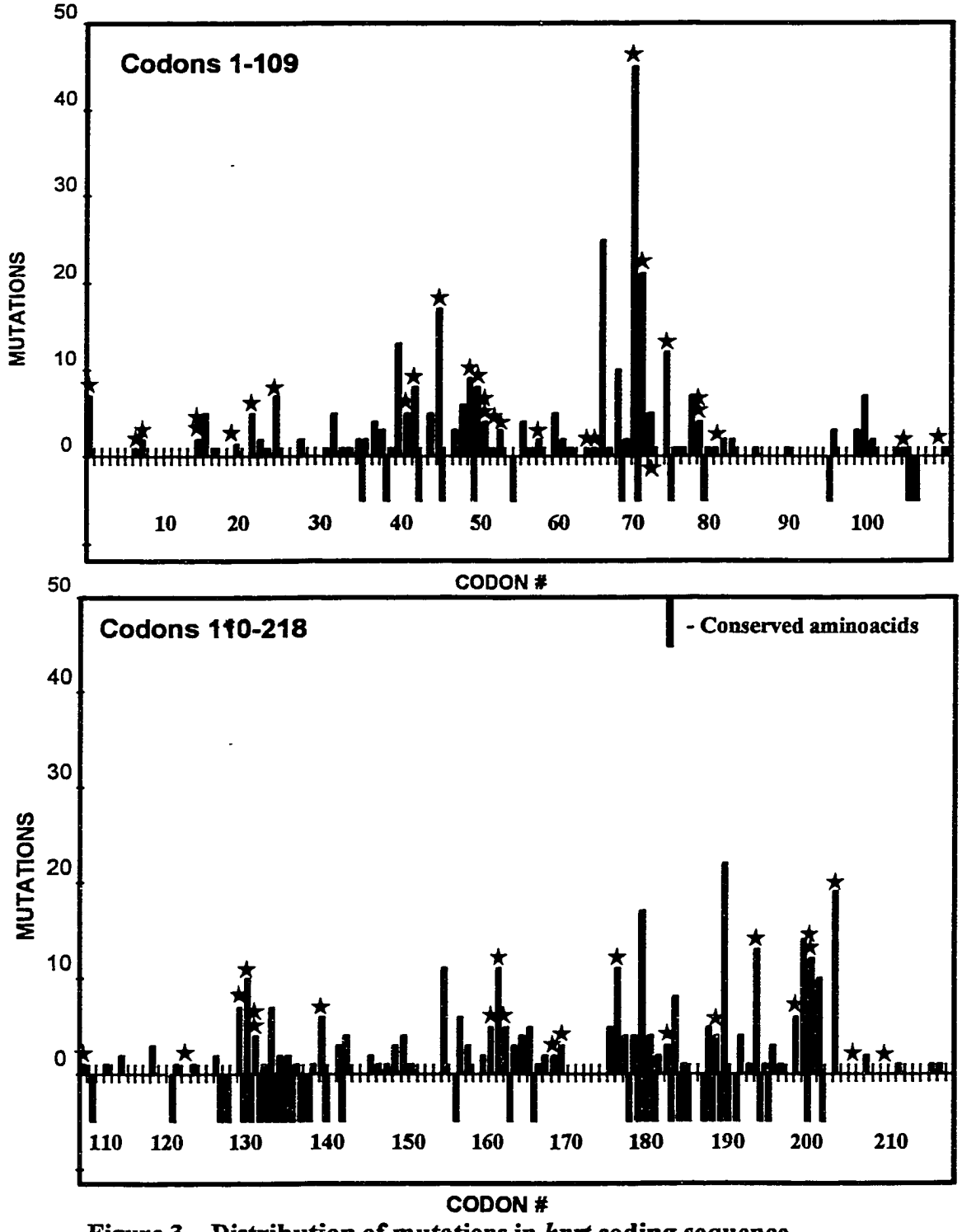

Figure 3. Distribution of mutations in *hprt* coding sequence
★ - mutation recovered in gout patients;
★ - mutations recovered in Lesch-Nyhan patients.

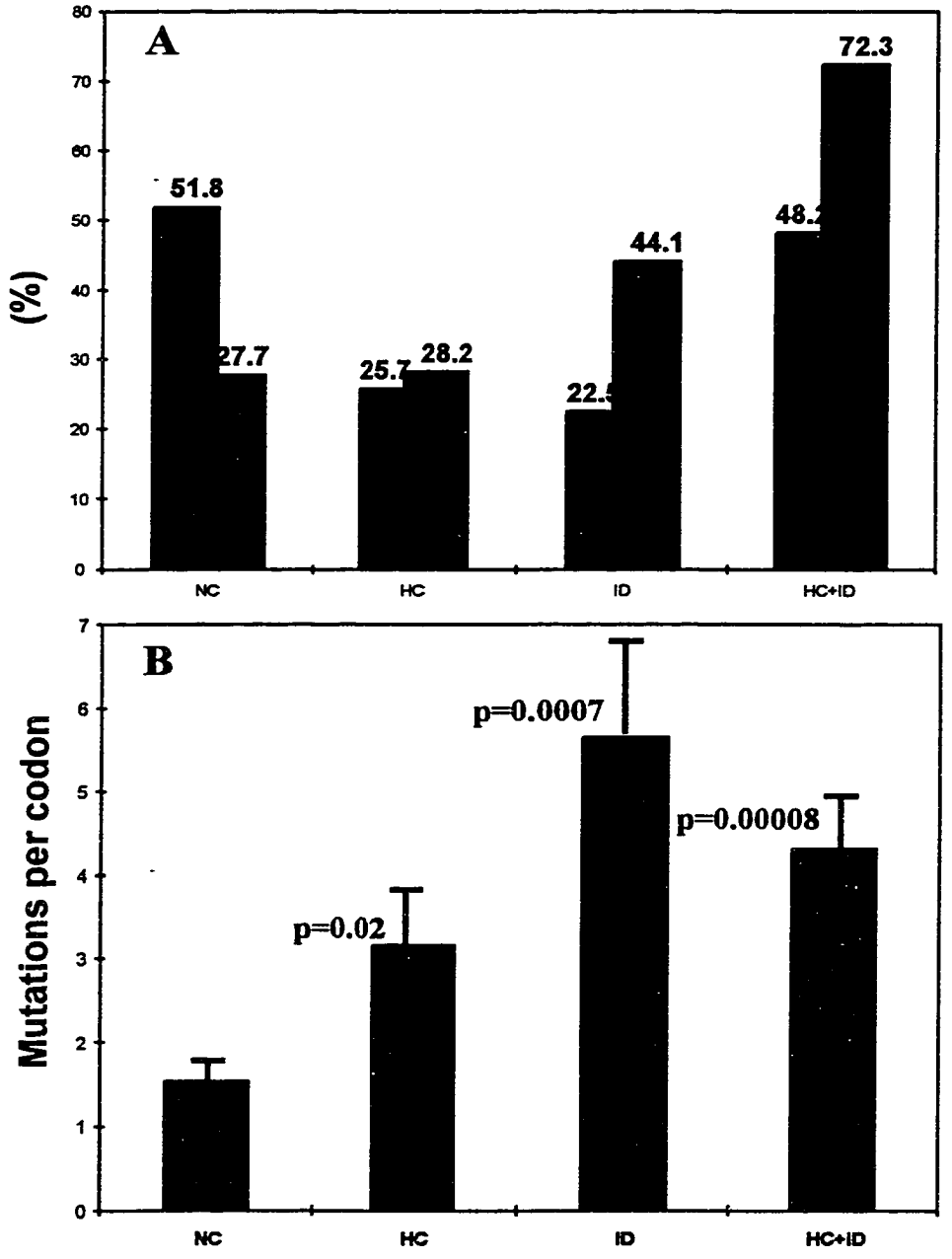

Figure 4. Comparison between conserved and non-conserved regions in *hprt*.
NC - non-conserved; HC - highly conserved; ID - identical (ID) residues;
Panel A: ■ - percentage in the sequence; ■ - percentage of mutations;
Panel B - Mutations per codon.

CHAPTER II - Possible Factors Leading to a Misjudgement of Mutant Frequencies in the *HPRT* Assay.

1. Abstract

Interactions between Cloning Efficiency (CE) and Mutant Frequency (MF) in the *HPRT* clonal assay in *in vitro* study were analysed. In 12 separate reconstruction experiments with independent pairs of Wild Type (WT) and Mutant (*HPRT*) clones, the CE of WT cells (Group 1) and the recovery of mutant cells in absence (Group 2), as well as in the presence of non-irradiated (Group 3), or irradiated (Group 4) WT cells (10^4 cells/well) was determined. The plating of mutant cells with irradiated WT cells improved their CEs by almost 30%. In contrast, the presence of non-irradiated WT cells led to a slight decline (10%) in CE of mutant cells, resulting in a significant difference between groups ($p = 0.0083$). The extent of decline in survival of mutant cells in the presence of non-irradiated WT cells negatively correlated ($r = 0.3496$, $p < 0.05$) with the initial CE of WT cells. The data suggest that the presence of WT cells in the selection plates may suppress the recovery of mutants in *HPRT* assay, and this negative effect is stronger in samples with high CE. These findings indicate a possible source for a serious underestimation of mutant frequencies (3-fold in the range of CEs from 10% to 60%) in the *HPRT* assay and may be useful for the interpretation of results from studies on exposure to mutagens in humans.

2. Introduction

The *hprt* clonal assay (Albertini *et al.*, 1982; Morley *et al.*, 1983) is extensively used in human and animal studies in order to estimate risks of environmental and occupational exposure to diverse mutagens (O'Neill *et al.*, 1990; Cole and Skopek, 1994; Branda *et al.*, 1993). In this context, the reliability and accuracy of the assay are important and have been the subject of several studies in different laboratories. One phenomenon, which has been identified, is an inverse relationship between overall cloning efficiencies (CEs) of the samples and mutant frequencies (MFs). According to estimates from several groups based on the analysis of hundreds of samples, a doubling of the CE results in a 24-47% decrease in MF (Cole *et al.*, 1988, 1991; Davies *et al.*, 1992; Tates *et al.*, 1991; Branda *et al.*, 1993; Dubeau *et al.*, 1994; Hou *et al.*, 1995). This observation is significant as it clearly reflects a recovery bias, although it is unknown whether this bias occurs at the higher or lower CEs.

One possible reason for the negative correlation between CE and MF is the difference in the density of T-cells in nonselective (-TG) and selective (+TG) 96-well plates (2-3 *vs.* 10^4 per well). It may appear that growth conditions are better in the selection plates (Albertini, 1985),

due to the presence of a large number of T-cells releasing an assortment of mitogens and nutrients into the medium.

Differing clonal potentials of T-cell subsets in the *HPRT* assay may also play a role (Cole *et al.*, 1988). In a recent study (Dubeau *et al.*, 1994) it was shown that the CE in nonselective conditions is strongly correlated with the fraction of CD4 (Helper) T-cells in the sample, whereas the CE in selection plates does not show this dependence. Moreover, CD4 clones are overrepresented under both conditions, however in selective plates, the CD4/CD8 ratio is 3-fold lower (9:1 *vs.* 3:1). The growth effect on CD8 (cytotoxic/suppresser) T-cells in selective plates is explained by their more complex requirements for activation. This, along with the high variability of CD4/CD8 ratios from sample to sample, may contribute to the inverse CE-MF relationships observed.

On the other hand, some studies have suggested that the more favourable growth conditions in selective plates and variation in T-cell subsets ratios may not be the only factors determining consistently different cell recovery under (-TG) and (+TG) conditions. For example, the inverse CE-MF relationship was observed even in split samples, where no significant shift in T-cell subsets can be expected (Cole *et al.*, 1988). Also, 10^4 wild-type (WT) T-cells present per well in the selective plates are poisoned by 6-thioguanine (6TG) while undergoing mitogen-induced proliferation. The killing of these cells may result in the release not only of mitogens and nutrients, but also cytotoxic factors. This is clearly the case in the experiment with sarcoma cell line STSAR-33, where conditioned medium from cells subjected to cytotoxic treatment (ionizing radiation, 5 Gy) produced cell killing in non-irradiated cultures (Hallahan *et al.*, 1989). This could be reversed by antibodies to Tumour Necrosis Factor α (TNFα).

The goal of the present study was to assess the possible effects of WT cells on recovery of mutants in selective plates.

3. Materials and Methods

In order to analyse interactions between mutant and WT T-cells in selection plates we designed the *in vitro* experiment described in Table 9. The purpose of this protocol was to separate possible active and passive factors affecting the recovery of mutants, and to estimate the extent of their influence with respect to the cloning efficiency of WT cells.

To reduce variability caused by the diversity of cells in a population, we carried out 3 series of experiments using 12 WT and 12 mutant clones independently derived from 4 samples taken from healthy volunteers being processed in the *HPRT* assay, according to a protocol detailed elsewhere (Curry *et al.*, 1993). Briefly, mononuclear cells (MNCs) were isolated from fresh blood samples using leukoprep tubes, and pre-incubated overnight in the growth medium without Interleukin-2 (IL-2) or feeder cells. MNC's were plated in selective or non-selective media in microtitre plates (96 well, flat bottom) at 10^4 and 3 cells per well respectively, along with 10^4 lethally irradiated (70 Gy) feeder cells (RJK 853 lymphoblastoid cells derived from a Lesch-Nyhan patient having a complete deletion of the *hprt* gene). Growth medium consisted of RPMI 1640 (Hyclone), 20% HL-1(Ventrix), 5% calf serum (Professional Diagnostics), 5% human AB serum (Gibco/BRL), 5U/ml IL-2 (Cellular Products Inc.), 0.25 μg/ml PHA (Wellcome), 2 mM L-glutamine, 2 mM pyruvic acid, 100 units/ml penicillin, 100 μg/ml streptomycin (Sigma) and 4% Fungizone (Gibco). Selection plates contained 10^{-5} M 6-thioguanine (Sigma). Plates were incubated on a sloped shelf (5°) in a 5% CO_2, 37°C, humidified incubator for 14 days. Twenty-four hours prior to scoring, the plates were rotated 180° on the sloped shelf, then scored visually

under an inverted phase contrast microscope for wells that contained expanding colonies. *HPRT* mutant frequencies were determined as a ratio between cloning efficiencies in selective and non-selective conditions.

One pair of WT and mutant clones from the same sample was studied in each experiment, where the CEs of WT cells under standard conditions (Group 1), as well as mutant cells alone (Group 2), in the presence of 10^4 non-irradiated (Group 3), or lethally irradiated (70 Gy, Group 4) WT cells were determined.

Table 9. Design of Experiment

	Group 1	Group 2	Group 3	Group 4
WT cells (2.6/well)	+			
TG^R cells (2.6 /well)		+	+	+
RJK-cells (70 Gy, 10^4 /well)	+	+	+	+
Non-irradiated WT-cells (10^4/well)			+	
Irradiated WT-cells (70 Gy, 10^4/well)				+
6-Thioguanine ($2x10^{-5}$M)		+	+	+

RJK - RJK853, lymphoblastoid cells carrying a total deletion of *hprt* obtained from a Lesch-Nyhan patient (Yang *et al.*, 1984).

4. Results

The data on the CEs of WT and mutant clones under different experimental conditions are presented in Table 10. The CEs of mutant cells plated in the presence of lethally irradiated WT T-cells (10^4/well, Group 4) improved by about 30% ($p=0.153$) compared to the CEs of mutant cells plated only with RJK-cells (Group 2). At the same time, there was a slight decrease (10%) in the CEs of mutant cells plated with non-irradiated WT T-cells. Most importantly, however, there was a significant difference ($p = 0.0083$) between groups 3 and 4, which were identical except that

in the former case the WT T-cells were not lethally irradiated, but rather killed *in situ* due to 6-TG poisoning.

Table 10. Cloning efficiencies of wild type (WT) and mutant (M) clones under different experimental conditions.

	CLONING EFFICIENCY (%)			
Experiment	**Group 1 (WT)**	**Group 2 (M)**	**Group 3 M+WT(0Gy)**	**Group 4 M+WT (70Gy)**
1	11.1	17.1	11.9	18.4
2	7.5	3.8	3.4	3.4
3	4.0	6.5	3.6	4.2
4	11.7	20.6	1.8	14.7
5	26.5	9.4	11.4	22.4
6	8.7	10.7	12.2	20.0
7	38.4	10.7	8.2	15.3
8	19.3	47.7	58.1	80.2
9	21.8	22.5	20.0	26.7
10	19.4	4.9	0.4	6.8
11	7.0	3.8	11.1	8.5
12	17.1	39.4	33.7	32.3
Mean ± SE	**16.0 ±2.8**	**16.4±4.1**	**14.7 ±4.7**	**21.1 ±6.0**
		100%	**89.6%**	**128.7%**
t-test (p =)*	**Group 2**		**0.417**	**0.153**
	Group3			**0.0083**

(*) - Student's t-test for paired samples, two tail;

Plotting changes in the CE of mutant clones in Group 3 compared to Group 4 (100%) versus the CEs of WT clones revealed a relatively strong negative correlation ($r = -0.3496$) significant at $p < 0.05$ (Figure 5). Interestingly, a similar but positive correlation ($r = +0.3230$, $p < 0.05$) was found between Group 2 (only RJK cells) and Group 4 (+irradiated WT-cells), when changes in CE (using Group 2 as 100%) were plotted against the CEs of WT clones (Figure 6).

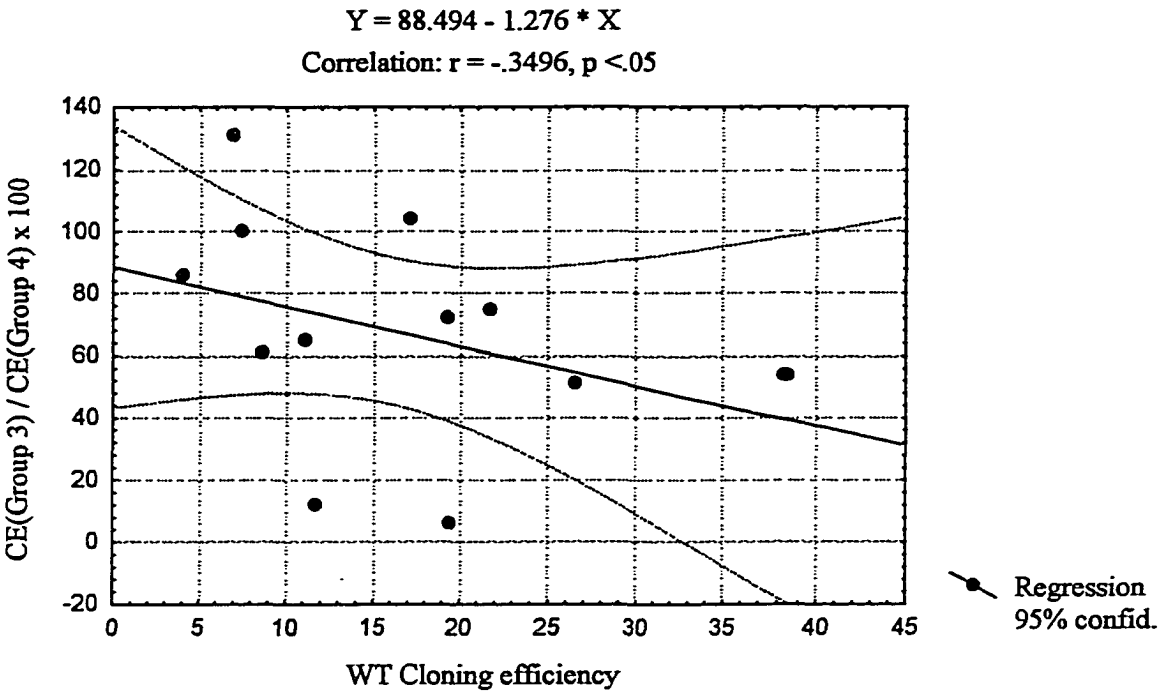

Figure 5. Influence of non-irradiated WT cells on survival of mutant cells
X-axis - initial CE of WT-clones; Y-axis - Percent of decline in CE of mutant cells plated with non-irradiated WT cells (Group 3) compared to CE of mutant cells plated with irradiated WT cells (Group 4). Solid line - line of best fit; Dotted lines - 95% confidence limits;

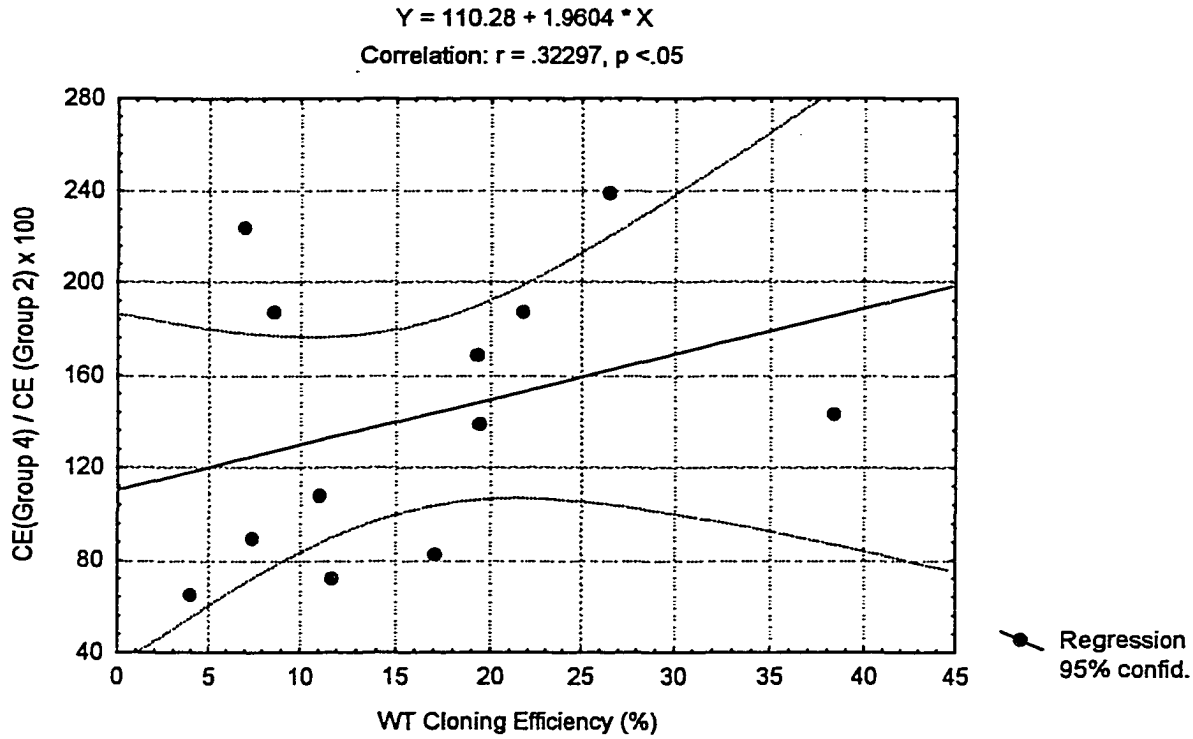

Figure 6. Influence of lethally irradiated WT cells on survival of mutant cell
X-axis - initial CE of WT-clones; Y-axis - Percent of increase in CE of mutant cells plated with irradiated WT cells (Group 4) compared to CE of mutant cells plated alone (Group 2). Solid line - line of best fit; Dotted lines - 95% confidence limits;

5. Discussion

The positive influence of an increased density of feeder cells on CE has been reported earlier (e.g., Hakoda *et al.*, 1988). These results are consistent with our data, where an increase in the CE of mutant cells plated in the presence of lethally irradiated WT cells was observed in 8 of 12 cases. The level of enhancement ranged from 1.2 to 2.4-fold (Table 10). The correlation between the improvement in clonal fitness of mutant cells and the CE of WT clones suggests that initial vitality of feeder cells (possibly expressed as concentration of growth factors, mitogens etc.) at least partially determines their supplemental value.

The presence of non-irradiated WT cells in selective plates abolishes this effect (Table 10, Fig. 6). These findings coincide with earlier reports, where increased cell density in *HPRT* assays led to a decline in recovery of mutants in different cell types, including T-lymphocytes (O'Neill *et al.*, 1987), Chinese hamster cells (Nishi *et al.*, 1984), and fibroblasts (van Zeeland and Simons, 1976). Similarly, dispersion of cells after a period of phenotypic expression prior to their transfer to selection medium resulted in a dramatic increase in MFs in Chinese hamster cells (Myhr and Dipalo, 1975). This may be accounted for by the metabolic co-operation between WT and mutant cells, and depletion of growth medium, because the WT cells remain alive for a few days in selection plates, and respond to mitogenic stimulation. It seems reasonable to assume that the higher the initial CE of WT cells in selective plates, the more nutrients and mitogens they consume before their elimination, and the greater their negative impact on the survival of the co-cultured mutant cells. Active mechanisms may also be involved as heavily-damaged WT cells can release cytokines that initiate apoptosis and thereby also depress the survival of mutant cells (Hallahan *et al.*, 1989).

In both data sets the same groups of mutant clones fell inside the 95% confidence limits. This may be partially explained by different requirements for CD4 and CD8 subsets for mitogenic activation. Promotion of growth of CD8 cells via interactions with WT cells may be greater, than negative effects of WT cells in selection plates, so that this fraction of mutant clones may respond differently.

Our result supports the notion that lethally irradiated T-cells serve as an additional source of nutrients, and can somewhat improve the survival of mutant cells in selection plates. In contrast, non-irradiated WT T-cells fail to realise their potential nutritional value and actually depress the recovery of mutants. The extent of this inhibition correlates with the initial viability of WT cells. We conclude that at low CE, WT cells have minimal influence on the survival of mutant cells. Therefore, MFs obtained from the samples with low CEs probably reflect actual values more accurately. In contrast, at high CEs the inhibitory effect of WT cells is quite pronounced and may result in a serious underestimation of MFs (up to 3-fold in the range of CEs from 10% to 60%.). These findings contradict the notion that MF values are biased at the low end of the CE scale (Albertini, 1985) , and these samples should be excluded from the analysis. The present data may be useful for the re-evaluation of mutagenicity studies in humans after environmental, occupational or therapeutic exposure to different mutagens, because the reported increase in MFs in exposed subjects may be at least partially accounted for by the commonly observed drop in CEs in these groups (Messing and Bradley, 1985; Dubeau *et al.*, 1994).

At the same time, conclusions derived from this experiment may not be directly applicable to *in vivo* studies. In *in vivo* studies the majority of T-cells are in G_0 phase of the cell cycle at the moment of plating, while in our experiment WT and mutant clones were in Log phase. This implies that *in vivo* T-lymphocytes enter S phase with a certain delay , so that period of active

interaction between WT and mutant cells before elimination of WT cells may be shorter which in turn may result in less pronounced consequences.

CHAPTER III - Molecular Analysis of Mutations in T-lymphocytes From Experienced Soviet Cosmonauts

1. Abstract

Somatic mutation in five cosmonauts who have completed spaceflights of 7 to 365 days was analysed using the clonal *HPRT* assay. The doses received in space by the cosmonauts ranged from 4 to 127 mGy. *Hprt* mutant frequencies were 2.4-5.0-fold higher than age-corrected values established for healthy, unexposed subjects in western countries (Tates *et al.*, 1991; Branda *et al.*, 1993), and 2-3-fold higher than those determined for unexposed individuals residing in Russia (Jones *et al.*, 1995). A total of 107 collected mutant clones were analysed by multiplex PCR (mPCR). No excess of deletions was detected and their frequency did not correlate with either accumulated dose or the age of the cosmonauts. In 62 mutants cDNA was isolated by RT-PCR for 62 mutants and sequenced. Those with splicing errors, as well as the mutants that did not produce cDNA, were further analysed by the sequencing of exon(s)-containing fragments amplified from genomic DNA. The mutational spectrum recovered from the cosmonauts differed substantially from that of unexposed healthy subjects ($p=0.042$), and exhibited an increased incidence of splicing errors, frameshifts, and complex mutations. Higher frequencies of contribution of AT>GC transitions and GC>TA transversions were also observed. The increased mutant frequencies and observed shifts in mutational spectra likely indicate a combination of potential influences including environment, life style, and occupational exposures. Further elucidation of these potential influences will require a more extensive study involving the general population sharing similar environment, cosmonaut in training and cosmonauts participating in space flights.

2. Introduction

Radiation has long been recognised as a potential peril of space travel. Astronauts risk exposure to diverse types of radiation contributed by galactic cosmic radiation, solar particle radiation, and trapped particle radiation. Crew members in a typical earth orbit receive approximately 0.01 - 1.0 mGy d^{-1}, depending upon the precise orbit, shielding, and the level of solar activity. It is estimated that an accumulated dose for a 90-day mission would be 3 to 7.5 cGy (NCRP report, 1989). Longer sojourns in space, the possibility of interplanetary travel, and the fact that astronauts now begin their space careers at younger ages, all accentuate the need to understand the effects of radiation in space.

In addition, ionizing radiation experienced in space is likely far more damaging than the conventional low linear energy transfer (LET) radiation common on earth. The relative biological effectiveness (RBE) of high-LET radiation is much greater, likely reflecting the lower efficiency of rejoining of double-strand breaks induced by high-LET radiation (Cole *et al.*, 1975; Blocher, 1988). For example, in experiments with human lymphocytes (Wolff *et al.*, 1991), the frequency

of chromatid aberrations induced by α-particles was 6 - 41 fold higher than the frequency of aberrations observed after exposure to X-rays. Moreover, individuals working in space are simultaneously subjected to both chronic and fluctuating exposures to high-LET radiation. There is evidence that chronic exposure to high-LET radiation results in enhanced levels of mutant induction and oncogenic transformation, compared to what is observed following acute exposure to the same dose (Brenner and Hall, 1990). According to Rossi and Kellerer (1986) this "inverse-dose-rate effect" reflects the cell-cycle specific radiosensitivity of exposed cells. At the same time, even small doses of high-LET radiation (in contrast to low-LET radiation) , may produce genetic effects because of the magnitude of their energy deposition.

Other factors possibly contributing to relative potency of protracted exposure are that exposure to very low doses of radiation does not cause cell cycle arrest, which might explain both enhanced radiosensitivity of mammalian cells (Koch *et al.*, 1973; Marples *et al.*, 1994), and elevated mutation formation in survived fraction. Alternatively, due to increased DNA damage, acute exposure can trigger protective mechanisms, resulting in more efficient repair, cell arrest and initiation of apoptosis in the heavily damaged cells. Because of this, the fraction of cells actually contributing to mutagenesis and carcinogenesis will likely decrease.

According to data obtained in several experiments aboard spacecraft (Problems of Space Biology, 1989), microgravity, or probably other factors constituting environment in the ship, may have a certain modifying effect on the genetic consequences of ionizing radiation in space. For example, compared to terrestrial control, exposure of plant cells to 30 Gy of γ-radiation prior spaceflight (Akoyev *et al.*, 1978), resulted in yields of cells with chromosomal aberrations (0.3-2.7-69.7%), which correlated with the duration of flights (7-18-27 days, respectively). This may in some way be related to the effects of microgravity.

This report describes the mutant frequencies and the molecular characterisation of *hprt* mutations in the peripheral T-lymphocytes of five soviet cosmonauts who have flown space missions of different duration.

3. Materials and Methods

3.1 Tissue culture

Fresh blood samples were obtained in the second half of 1992 from five cosmonauts who have completed spaceflights of varying duration and exposure to radiation (Table 12). Doses received by cosmonauts ranged from 0.4 to 12.7 cGy. Mononuclear cells (MNCs) were isolated using the ficoll-paque technique, counted and diluted to 10^7 cells ml^{-1} in 50% calf serum (Professional Diagnostics), 10% DMSO (Sigma), and 40% RPMI 1640 (Hyclone) and then frozen by the styrofoam box method (Cole *et al.*, 1988) in a -80°C freezer. The samples were shipped to Canada on solid CO_2 and then stored in liquid nitrogen until required.

The MNC's were thawed, washed twice in RPMI 1640 supplemented with 10% Calf Bovine Serum (CBS), and pre-incubated in non-selective medium (Cole *et al.*, 1988) overnight. MNC's were plated in 200 µl of selective and non-selective media in microtitre plates (96 well, flat bottom) at 10^4 and 3 cells per well respectively, along with 10^4 lethally irradiated feeder cells (RJK 853 lymphoblastoid cells derived from a Lesch-Nyhan patient having a complete deletion of the *hprt* gene). Growth medium consisted of RPMI 1640 (Hyclone), 20% HL-1(Ventrix), 5% calf serum (Professional Diagnostics), 5% human AB serum (Gibco/BRL), 5U/ml IL-2 (Cellular Products Inc.), 0.25 µg/ml PHA (Wellcome), 2 mM L-glutamine, 2 mM pyruvic acid, 100 units/ml penicillin, 100 µg/ml streptomycin (Sigma) and 4% Fungizone (Gibco). Selection plates contained 10^{-5} M 6-thioguanine (Sigma). Plates were incubated on a sloped shelf (5°) in a 5%

CO_2, 37° C, humidified incubator for a period of 14 days. Twenty-four hours prior to scoring, the plates were rotated 180° on the sloped shelf then scored visually under an inverted phase contrast microscope for wells that contained expanding colonies. *Hprt* mutant frequencies were determined as a ratio between cloning efficiencies in selective and non-selective conditions. Mutant colonies were transferred to a 24 well flat bottom dish with 2 ml of fresh selective media and expanded for the analysis of genomic DNA.

3.2 PCR reactions and sequencing

Amplification of the 8 fragments from genomic *hprt* sequence (multiplex PCR analysis) containing all 9 exons plus some flanking intronic sequences was performed essentially as described by Gibbs *et al.* (1990). Crude cell extracts preparation from about 10^4 cells per reaction was carried out as described by Fuscoe *et al.* (1992a). Amplification of *HPRT* cDNA from cellular mRNA (RT-PCR assay) of mutant clones was carried out in two consecutive PCR reactions using nested primers (Yang *et al.*, 1989).

Recovered cDNAs were sequenced on a Pharmacia A.L.F. automated sequencer according to the manufacturer's instructions. In those *HPRT*⁻ mutants that did not yield products in RT-PCR assay or produced cDNA with exon exclusions, genomic fragments containing exons were amplified separately, and also sequenced using primers as in (PCR: A practical approach, 1991). In some cases mutations were positioned too close to the primer site, and these exons were sequenced using our own primers (Table 11). Eight mutants with wild-type mPCR patterns did not produce cDNA and the amount of available genetic material was very limited, so that separate amplification and sequencing of all exons could not be performed and mutations were not determined.

Mutational spectra were compared using the Monte Carlo test (Adams and Skopek, 1987) and comparison of specific types of mutations was performed via the chi-square test using Statistica (Statsoft) software.

Table 11. Additional fluorescent forward primers used for direct sequencing of *HPRT* exons

EXON	POSITION	SEQUENCE (5'–>3')	T_m
3	16629-16646	TGAAGGAGATGGGAGGCC	51.9
5	31446-31463	GCTTCCAAATCCCAGCAG	50.3
7	39756-39773	CTCTTTTGTAATGCCCTG	42.6
8	39956-39973	TAGAGAGGCACATTTGCC	45.5

Positions of primers are shown according to Edwards *et al.*, 1990

Table 12. Flight experience and radiation exposures of cosmonauts

SUBJECT	AGE * (YRS)	FLIGHT DURATION (DAYS - YEAR)	DOSE** (cGy)
SP5	57	7 1969 7 1975 7 1980	0.1 0.1 0.2
SP6	52	9 1984 132 1990	0.1 3.9
SP7	39	180 1990	4.6
SP8	41	365 1988 168 1991	8.7 4.0
SP9	48	9 1982 2 1983 167 1990	0.1 0.05 5.7

(*) Age at time of sampling.
(**) Dosimetry data was obtained from the Dosimetry Division of the Institute for Biomedical Problems, Moscow, Russia

4. Results

Mutational data from the *HPRT* T-cell assay from the five cosmonauts are presented in Table 13. Cloning efficiencies of the T-cells under the non-selective conditions ranged from 18 to 29% while mutant frequencies (MF) ranged from 21 to 58 x 10^{-6}. These MFs are considerably higher than values for healthy, unexposed subjects of matching age in Western countries (Tates *et al.*, 1991; Branda *et al.*, 1993), and individuals residing in Russia (Jones *et al.*, 1995).

The majority of the multiplex PCR (mPCR) patterns of the *hprt* mutations were normal, *i.e.*, all 8 exon fragments were present, and normal in size. Some of the patterns revealed the loss or the shifting of bands indicating intra- or inter-genic deletion events. Gross alterations, as detected by mPCR, depending upon the subject, accounted for 0-21.4% of all mutations. Incidence of deletions slightly exceeded levels established for spontaneous mutational spectrum (Albertini *et al.*, 1992) only in one case (SP5), and no correlation was observed for gross rearrangements with accumulated dose, duration of flights or age.

Table 13. Cloning Efficiencies (CE) and Mutant Frequencies (MF) of cosmonauts

SAMPLE	Number of Mutants	CE_{-tg} (%)	CE_{+tg} x10^{-6}	MF x 10^{-6}
SP5	14	24.9	14.5	58.2
SP6	3	18.0	4.8	26.7
SP7	32	28.8	12.5	43.4
SP8	24	24.5	5.2	21.2
SP9	34	20.8	8.4	40.5

CE_{-tg} and CE_{+tg} – Cloning efficiencies in absence and presence of 6-thioguanine.

In total, 62 mutants were analysed at the cDNA sequence level (Table 14). Fifty-seven percent of the mutations involved splicing errors. The remaining included deletions (3.3%), double substitutions (3.1%), complex events (6.6%), and point mutations (26.2%). The point mutations consisted of (+) and (-) frameshifts (1.6 and 4.9% respectively) and base substitutions (19.7%), 11.5% of which were transitions and 8.2% were transversions. In 2 cases (SP6-1 and SP7-16), no mutation could be found in the coding sequence. The most common splicing errors involved the skipping of exons 2 and 3 (42.3%) and exon 8 (16.2%). In 6 cases (Table 16), changes in the mPCR pattern indicated partial of complete exon deletions. One apparently complex (at the pre-mRNA processing level) mutation, SP7-8, was recovered. This mutation consisted of the loss of exons 5 and 6 (cDNA) with the insertion of a 36 bp fragment originating from intron 4, while the mPCR pattern showed deletion of exon 6. An analysis of the sequences flanking the inserted fragment revealed the presence of both 5'- and 3'-cryptic putative splice recognition sites, suggesting a mis-splicing mechanism due to disappearance of the legitimate consensus splice sites. A similar mechanism is also proposed for the mutant SP7-13 with a partial deletion of exon 8, because the sequence preceding the deletion also includes a 3'- cryptic recognition site.

Twenty-five mutants that did not produce cDNA in RT-PCR reaction were analysed by the direct sequencing of exons amplified from genomic DNA (Table 15). The distribution of mutations by class in this group was somewhat different from the cDNA mutational spectrum (exon losses excluded), with increased fractions of frameshifts (20% *vs.* 15.3%) deletions (12% *vs.* 7.7%) and complex mutations (12% *vs.* 9.2%). In 2 mutants (SP5-1 and SP8-6), analysis of genomic DNA did not reveal any alteration in the coding sequence. A substantial fraction of

mutants in this group (36%) carried nonsense mutations, whereas in the cDNA spectrum only 11.3% of nonsense mutations were observed.

Further analysis of mutants with splicing errors at the genomic level (Table 16) showed that about two thirds of mis-spliced mutants (23/35) carried mutations in the splice sites, exons or deletions involving one or two exons. Fifty-two % of the elucidated splicing errors were caused by base substitutions in splice sites mostly involving invariant AG and GT nucleotides (35%). Deletions were the cause of mis-splicing in 34.8% (8) of cases. Half of them spanned or involved splice sites regions, and the rest were relatively large and resulted in elimination of one or two exons. Three remaining mutants carried base substitution in exon 2 (SP9-34, E2-3 loss), 5 bp duplication in exon 2 (SP9-25, E2-3 loss), and complex mutations consisting of base substitution at the end of exon 3 and deletion spanning 5'-splice site of intron 3 (SP8-15, E2-3 loss). In several mutants with skipping of exons 3 (1/3), 7(1/1), 8 (2/5), 2-3 (4/12) and 2-6 (3/4) analysis of intron-exon boundaries did not reveal any changes.

Thirty independent deletions were detected and 14 of them sequenced (Tables 14,15 and 16). The majority of deletions (64% -9/14) were flanked by direct repeats. In the rest of the deletion, mutants flanking sequences contained neither direct nor inverted repeats that might explain their origin. One of the deletions found in cDNA was relatively large, spanning approximately 25 Kb of genomic DNA from exon 2 to exon 8 (position 48 to 569 at the cDNA level), and was flanked by nearly perfect (two mismatches) 15 bp direct repeats. Deletions were also involved in the 7 out of 8 complex mutations and were accompanied by point mutations in the nearest vicinity (SP8-14, SP8-15), insertions (SP5-13, SP7-8, SP7-23, SP8-20), or unidentified mutation(s) causing exon skipping (SP9-9).

In total, 90 mutants were analysed at the cDNA and/or genomic DNA level, and in 66 cases data on the actual sequence context of mutations was obtained (Tables 14, 15 and 16). Analysis of mutational spectrum was performed on a dataset comprised of cDNAs and directly sequenced mutants (Tables 14 and 15), which despite some minor differences were essentially identical (p=0.73). The comparison of cosmonauts and background mutational spectra (Cariello *et al.*, 1994) revealed a statistically significant difference ($p = 0.0425$) between them (Table 17) with elevated incidence of splice errors and complex mutations in our dataset. Within base substitutions subsets frequencies of AT>GC transitions and GC>TA transversions in cosmonauts' samples were considerably higher (2.6- and 1.3-fold respectively, Table 18). Only 1 of the 8 recovered G:C->A:T transitions occurred at potential CpG sites, which are thought to be preferred sites for mutations when modified to 5'-methylcytosine (Rideout *et al.*, 1990). This is low compared to normal spontaneous data (25.3%).

Table 14. *Hprt* cDNA mutations recovered from cosmonauts

Mutant	POSITION	MUTATION	SEQUENCE CONTEXT	CHANGE
SP5-3	143	G–>A	GAAC..a..TCTT	Arg→His
SP5-4		E5 loss		
SP5-10		E2-8 loss		
SP5-14	185-193	Deletion	TCACA <ttgtagccc> TCTGT	
SP6-1		Intact cDNA	No change detected	
SP6-2		E2-3 loss		
SP7-2		E3 loss		
SP7-4		E4 loss		
SP7-5		E4 or 2-4 loss		
SP7-6		E3 or 2-3 loss		
SP7-7	599,600	GG–>AA	CTTCA..aa..GATTT	Arg→Lys
SP7-8	28707-28741*	E5-6 loss and insertion 36 b I4	TTGGTTGTTCAG<INSERT> GTATTT	
SP7-11		E8 loss		
SP7-12		E2-3 loss		
SP7-13	530-553	E8 part.loss	EX.7<ttgttggatttgaaattccag> ACAAG	
SP7-14		E7 loss		
SP7-15		E2-3, and 8 loss		
SP7-16		Intact cDNA	No change detected	
SP7-17		E4 loss		
SP7-18	118	G–>T	CTCAT..t..GACTA	Gly-Ter
SP7-19	203	T–>C	TGTGC..c..CAAGG	Leu-Pro
SP7-21	463,464	CC–>TT	ATAAT..tt..AAGAT	Pro-Leu
SP7-22	617	G–>A	TGTTT..a..TGTCA	Cys→Tyr
SP7-24	562	Missing G	TGTTT.. ..TTGTA	
SP7-25		E2-3 loss		
SP7-26		E8 loss		
SP7-29		E2-6 loss		
SP7-30		E2-3 loss		
SP7-32	2	T–>C	A..c..GGCGA	Met→Thr

Table 14. Continued

SP8-2		E2-3 loss		
SP8-4	597	C–>A	TACTT..a..AGGGA	Phe-Leu
SP8-5	384	Missing G	GGAAA.. ..gtatg	
SP8-9		E3 or 2-3 loss		
SP8-10	361	G–>A	GAGAT..a..ATCTC	Asp→Asn
SP8-14	48-569 612-618	Deletion and 2 or 3 point deletions	ACC<aggttatgaccttga..tgt> AGGATATGCCCTTGA TGTTTG–>TTT	
SP8-15		E2-3 loss		
SP8-16	404	A–>T	aaagg..t..TATAA	Asp→Phe
SP8-17		E2-6 loss		
SP8-18	210	Extra G	GGGGGG–>GGGGGGG	
SP8-19	616	T–>C	ATGTT..c..GTGTC	Cys→Arg
SP8-21	550	C–>T	AATTT..t..TAGAC	
SP8-23	78	Missing T	CCTAA.. ..CATTA	
SP9-2		E2-8 loss		
SP9-3	508	C–>T	CCCCA..t..GAAGT	Arg-Ter
SP9-4		E3 or 2-3 loss		
SP9-5	410	T–>A	TATAA..a..TGACA	Ile→Asn
SP9-6		E3 loss		
SP9-7		E3 loss		
SP9-9	465-477	E2-3 loss and deletion	TAATCC<aaagatggtcaag> GTCGC	
SP9-10		E8 loss		
SP9-14		E2-3 loss		
SP9-16		E2-6 loss		
SP9-18		E2-3 loss		
SP9-19	221	T–>G	TAAAT..g..CTTTG	Phe-Cys
SP9-20		E8 loss		
SP9-21		E5 loss		
SP9-25		E2-3 loss		
SP9-28		E2-6 loss		

Table 14. Continued

SP9-30	388	G–>T	agAAT..t..TCTTG	Val–>Phe
SP9-31		E2-3 loss		
SP9-32		E8 loss		
SP9-33	162-180	Deletion	TGTG<atgaa...aggcc> ATCACATT	
SP9-34		E2-3 loss		

Position of mutations is given according to Jolly *et al.*, 1983; Direct repeats flanking deletions and putative splice consensus sites are underlined.

Table 15. Mutations detected by direct sequencing of exons amplified from genomic DNA.

Mutant	Position	Mutation	Context	Change
SP5-1		No change		
SP5-2	40061	Extra T	CAAGT..T..TTGTT	ttt/tgt tgt agg ata tgc cct TGA
SP5-5	16762-16764	Missing T	TAGAT.. ..TTATC	gat/tta tca gac TGA
SP5-6	I8:-1	G→C	ttata..c..CATGT	
SP5-8	34989	C-->T	TCAGG..t..AGTAT	Gln-->Ter (TAG)
SP5-13	14813-14846	Deletion/ insertion	TGATT<TATT.. ..GGATT>TGGAA → TGATT-CATTA-TGGAA	gat/tca tta tgg aaa ggg tgt tta ttc ctc atg gac TAA
SP6-3	16676	G-->A	TCAAG..a..GGGGC	Gly-->Arg
SP7-1	39612-39824	E7 p.deletion	tgtct<gtagt...TGCTG>GTGAA	
SP7-3	14885	A→G	TGGAC..g..Ggtaag	Arg→Gly
SP7-9	27907-27910	Missing G	ACAGG.. ..GACAT	ggg/aca TAA
SP7-23	35013-35026	Deletion/ insertion	TCAAG <GTCGCAAGgtatgta> tgaca →TCAAG-TC-tgaca	
SP8-1	16679(211)	G→C	AGGGG..c..GCTAT	Gly→Arg
SP8-3	35010	A-->G	TGGTC..g..AGGTC	Lys-->Glu
SP8-6		No change		
SP8-12	39816	C-->G	aacag..g..TTGCT	Ser-->Arg
SP8-20	16775-16776	Deletion/ insertion	GACTG<aa>GAGCTA → GACTG-TCCCCTC-GAGCTA	ctg/ tcc cct cga gct att gta atg acc agt caa cag ggg aca TAA
SP9-1	I7:5	G→T	Tgtaa..t..tgaat	
Sp9-8	I8:-2	A-->G	tttat..g..gCATG	
SP9-12	38816	C→G	aacag..c..TTGCT	Ser→Arg
SP9-13	16691(224)	Missing T	ATTCT.. ..TGCTG	ttc/ ttg ctg acc tgc tgg att aca tca aag cac TGA
SP9-17	14865	C→G	TATTC..g..TCATG	Pro→Arg
SP9-24	40040	G-->A	TGTTG..a..ATTTG	Gly-->Glu
SP9-26	41497 (652)	Missing G	ACAAA.. ..CCTAA	
SP9-27	16736-16744	Deletion	TAGTG<ATAGATCC>ATTCC	agt/gat tcc tat gac tgt aga ttt tat cag act gaa gag cta ttg TAA
SP9-29	35000-35012		AATCC <AAAGATGGTCAAG> GTCGC	aat/ ccg tcg caa gct tgc tgg TGA

Legend: In Tables 15 and 16 positions of mutations are given according Edwards *et al.*, 1990. Direct repeats flanking deletions are underlined. Lower case letters represent base substitutions, or in case of deletions, intron sequences involved. Upper case letters represent exon sequences.

Table 16. Analysis of misspliced *HPRT*⁻ mutants

MUTANT	Missing exon(s) cDNA	GENOMIC SEQUENCE		
		MUTATION	POSITION	CONTEXT
SP5-4	E5	A→G	I4:-2	cttct..a..gAATG
SP7-2	E3	E3 - WT		
SP7-4	E4	E4 - WT		
SP7-5	E4 - E2-4	E4 - p.deletion	27952-27972	TAACT <GGAAAGg.. ..tgaaa>gggaag
SP7-6	E3 - E2-3	E3 deletion		
SP7-8	E5-6 + ins.	E6 deletion		
SP7-11	E8	T→G	I7:-13	aatta..g..gattctttttagTTG
SP7-12	E2-3	E2,E3 - WT		
SP7-13	E8 partial	E8 - WT		
SP7-14	E7	E7 - WT		
SP7-15	E2-3 and E8	E2,E3 -WT G→A	I8:+1	TGATT..a..taagt
SP7-17	E4	E4 - WT		
SP7-25	E2-3	G→T	E3:+5	gGACT..t..AACGT
SP7-29	E2-6	E2,E6 - WT		
SP7-30	E2-3	G→T	E3:+5	gGACT..t..AACGT
SP8-2	E2-3	E2-3 deletion		
SP8-9	E2 or E2-3	A-->G	I2:-2	tctgt..g..gGACT
SP8-15	E2-3	A→C and Deletion	16769;16773-16804	TTATC..c..GAC <tgaag…atata> tgatt
SP8-17	E2-6	E2,E6 - WT		
SP9-2	E2-8	E2 p.deletion	14785-14843	ATT<AGTGA…TGCTG>AG GATTT
SP9-4	E3 or 2-3	G→A	I2:-1	ctgta..a..GACTG
SP9-6	E3	A→G	I2:-2	tctgt..g..gGACT
SP9-7	E3	A→G	I2:-2	tctgt..g..gGACT
SP9-9	E2-3	E2,E3-WT: Deletion (E6)	35000-35012	AATCC<AAAGATGGTCAA G>GTCGC
SP9-10	E8	E8 - WT		
SP9-14	E2-3	E3 p. deletion	16759-16817	TTATC <AGACT…tttag> tggca
SP9-16	E2-6	G→C	I5:-1	tgaaa..c..GATAT
SP9-18	E2-3	G→A	I2:-1	ctgta..a..GACTG
SP9-20	E8	I7 p. deletion	39977-40016	gcagt <attag… gatga> attat
SP9-21	E5	E5 deletion		
SP9-25	E2-3	Duplication	14836-14840	CATTA-TGCTG-TGCTG-AGGAT
SP9-28	E2-6	E2,E6 - WT		
SP9-31	E2-3	E2,E3 - WT		
SP9-32	E8	G→T	I8:+5	Tgtaa..t..taatt
SP9-34	E2-3	G→T	14840 (E2)	ATGCT..t..AGGAT

Legend: Format as in Table 15.

Table 17. Spectrum of mutations recovered from cosmonauts compared to spontaneous spectrum from *hprt* database (Cariello *et al.*, 1994).

MUTATIONS	COSMONAUT		SPONTANEOUS	
	N	%	N	%
BASE SUBSTITUTIONS	**26**	**30.2**	**215**	**46.3**
SPLICE ERRORS	37	43.0	149	32.1
TANDEM	2	2.3	4	0.9
FRAMESHIFTS (+)	2	2.3	8	1.7
(-)	7	8.1	24	5.2
DELETIONS	5	5.9	41	8.8
INSERTIONS	0	0	4	0.9
COMPLEX	7	8.1	19	4.1
TOTAL	**86**	**100**	**464**	**100**

Legend: In tables 17 and 18, the cosmonaut dataset is comprised of cDNA mutants and mutants directly sequenced from genomic DNA.

Table 18. Spectrum of base substitutions recovered from cosmonauts compared to spontaneous spectrum from *hprt* database (Cariello *et al.*, 1994).

BASE SUBSTITUTIONS		COSMONAUTS		SPONTANEOUS	
		N	%	N	%
TRANSITIONS	GC>AT	8	30.8	79	36.7
	AT>GC	6	23.1*	19	8.8
TRANSVERSIONS	GC>TA	4	15.4	25	11.6
	GC>CG	5	19.2	33	15.4
	AT>TA	2	7.7	27	12.6
	AT>CG	1	3.8	32	14.9
TOTAL		**26**	**100**	**215**	**100**

Note: (*) - $p = 0.05$ (chi-square test).

5. Discussion

5.1 Mutant frequencies:

Hprt mutant frequencies determined in cosmonauts samples were 2.4- to 5.0-fold higher than the age-corrected values established for healthy unexposed subjects (Tates *et al.*, 1991; Branda *et al.*, 1993). We note, however, that the direct comparison of cosmonauts' MF data with data collected in North America and Western Europe is probably not entirely justified. The considerable differences in the levels of pollution, diet and life style in former Soviet Union and in the West could affect mutation. Unfortunately, samples from age- and life-style matching controls were not available for this study. Nonetheless, we can refer to data generated in our laboratory on 7 pairs of Russian twins (Curry *et al.*, 1997) showing that their mutant frequencies are considerably higher than in Western control (16.6 ± 8.2). Similar results were obtained in a recent study on age-correlated increase of *HPRT*⁻ mutant frequencies in groups of individuals from the United States of America and Russia (Jones *et al.*, 1995). Russians of unknown smoking status showed 2-fold higher MFs than age-matched smokers in the United States of America. This suggests that life-style factors in the former Soviet Union may, at least partially, account for the elevated mutant frequencies in the cosmonauts' samples. However, MFs in cosmonauts' samples are still 2-3-fold higher than age-matched Russian controls, which indicates that some additional factors, possibly occupation related, may be involved.

5.2 Molecular analysis:

5.2.1 cDNA:

Forty-three percent of the recovered cDNAs were incorrectly spliced. Splicing errors involved all exons with the exception of exons 1 and 9. Observed percentage of splicing errors was somewhat higher than reported by other groups (30%-Recio *et al.*, 1990: 35%-Rossi *et al.*, 1990a: 39%- Rossi *et al.*, 1990b), and that found in the *hprt* database of spontaneous mutations (32%). The fact that actual mutations in a considerable fraction of mutants remain obscure makes exon skipping a confounding factor for the purposes of analysis of mutational spectrum. The molecular analysis of splice junctions revealed different types of mutations. Apart from splicing errors caused by deletion of entire exons, 27.8% (5) of mutations were located at 5'-splice sites, 44% (8) - at 3'splice sites, and 28% (5) - within exon sequences. 7 cDNAs carried exclusions of more than two exons and most of them involved exons 2-6. Similar events were observed by other groups (Steingrimstottir *et al.*, 1992; Meninchini *et al.*, 1994; Valentine and Heflich, 1995) in *hprt*, and other genes (Carothers *et al.*, 1993). This phenomenon is explained by the clustering of exons at the initial step of splicing, thereby a single mutation may lead to exclusion of several exons (Carothers *et al.*, 1993). In 11 splice mutants no mutations could be found either in splice sites or exon sequences. This indicates that some additional sequences located elsewhere may be important for accurate splicing.

5.2.2 Directly sequenced mutants:

In about 30 mutants, cDNA could not be obtained. Direct sequencing of exons amplified from genomic DNA revealed a striking abundance of nonsense mutations (36%) in this set. It has been found that nonsense mutants carrying termination codons within internal exons show a 5-6-fold reduction in *hprt* mRNA steady-state level (Manjanatha *et al.*, 1994). All of the identified nonsense mutants in this group resulted in termination codons situated within internal exons, which may partially explain their failure to generate cDNA in RT-PCR assay.

5.2.3 Deletions

It has been proposed that exposure to ionizing radiation will lead to an increase in deletion events (Sankaranarayanan, 1991), presumably due to radiation-induced double-strand breaks. Such a shift was not reflected in the mutational spectrum observed (Tables 14-16). Indeed, including splicing mutations, about 90% of mutations recovered from the five cosmonauts were point mutations, which is very similar to a spontaneous *hprt* mutational spectrum observed by Albertini *et al.* (1990). In terms of radiation exposure, most experimental data reflects acute exposures to relatively high doses of radiation. Cosmonauts, on the other hand, are exposed to low doses of both protracted, and fractionated, high-LET exposures under conditions of microgravity. Consequently, cosmonauts receive a different range of damages and possibly differ with regards to induced DNA repair (Sankaranarayanan *et al.*, 1989). It is possible that the chronic doses are so low that the resulting level of damage is insufficient to activate the cell's protective mechanisms (Oftedal, 1991).

Upon low-dose exposure, indirect effects of ionizing radiation become prevalent (von Sonntag, 1987). There is evidence suggesting that mutations recovered after exposure to low doses of high-LET radiation are not the result of a direct interaction between the particle and DNA. For example, in the case of CHO cells exposed to low doses of plutonium-238 α-particle radiation (Nagasawa *et al.*, 1990), only in about 5% of the cells demonstrating radiation-induced sister chromatid exchanges, were the nuclei actually traversed by α-particles. It is thought that the vast majority of mutations induced by low-dose high-LET radiation reflect free radicals, so that the fraction of deletions may not be so much larger than observed spontaneously (Oller and Thilly, 1992). If this is the case, it may thus not be surprising that in terms of deletions the spectrum obtained in the cosmonauts does not differ greatly from that obtained in the controls

It has been found that radiation-induced deletions may have some distinctive properties. Compared to spontaneous control, breakpoints of induced deletions in an *aprt* gene, appeared to fall predominantly in A:T-rich regions (Meuth, 1992). In our study, T:A pairs were found at the breakpoints far more often than G:C pairs (61% *vs.* 39%), which seems consistent with Meuth's findings. However, analysis of spontaneous deletions in an *HPRT* database (Cariello *et al.*, 1994) revealed a similar ratio between A:T and G:C pairs (59% *vs.* 41%). This, along with the fact that A:T pairs are prevalent in the *hprt* coding sequence, (58.7%) suggests that there is no bias toward A:T pairs at the deletion breakpoints in our dataset.

The majority of recovered deletions were flanked by direct repeats, which is considered to be an indication of their spontaneous nature, (Krawzcak and Cooper, 1991) related to proposed slippage-misalignment mechanism during replication (Streisinger *et al.*,

1966). At the same time it is feasible to assume that slippage may occur, independent of replication following the induction of single or double strand breaks.

Three complex mutations exhibiting coinciding deletion-insertion events were identified in exons 2, 3 and 6. Similar mutations in the *hprt* gene were reported by other groups (Gibbs *et al.*, 1989; Ikehata *et al.*, 1989; Giver *et al.*, 1993), and were clustered within the exon 6 sequence. The mechanism of their formation is unclear and may involve two consecutive slippage-misalignment steps (Giver *et al.*, 1993). We also note that deleted nucleotides in our mutants tend to be replaced by sequences composed of mostly thymine, adenine, and cytosine, and almost never by guanine.

5.3 Mutational spectra

The shifts observed in the cosmonauts' mutational spectrum are mildly reminiscent of exposure to environmental mutagens. For example, G:C to T:A transversions found in moderate excess in the cosmonauts' mutational spectra are also typical for exposure to certain environmental and dietary mutagens such as benz[a]pyrene (Mazur and Glickman, 1988) and PhIP (Wu *et al.*, 1995). Also, alkylating agents, including mutagens specific for tobacco, (Gorman and Steinberg, 1994; Tiano *et al.*, 1994) induce G:C to A:T transitions at predominantly non-CpG sites. G to A mispairing might also occur upon formation of 8-oxo-7-hydrodeoxyguanosine (8-oxodG), which is produced by ionizing radiation through interaction of $OH^{\bullet}$ radicals with guanine (Dizdaroglu, 1985). At least in bacterial systems it has been shown that the proton-induced mutational spectrum is dominated by G:C to T:A transversions (Takimoto *et al.*, 1993). If this were also true for mammalian cells, this mechanism would be in agreement with predominance of secondary effects for mutagenesis

resulting from low doses of radiation. In addition, compound mutants (SP8-14 and SP8-15) may also reflect exposure, as their appearance is consistent with the locally multiply damaged sites (LMDS) model for particle-radiation induced DNA lesions (Brenner and Ward., 1992). In this model, LMDS are very localised energy depositions of 2-5 ionisations 1-4 nm in diameter, which roughly corresponds to 10-15 nucleotides. In at least one of these mutants (SP8-15), the distance between two mutations (4 bp) is within defined limits.

Other factors may also affect the observed mutational spectrum. For example, a potential contributing factor is the cosmonauts' exposure to oxygen-enriched environments, and routine, regimented exercise. It is shown that physical exercise, above the aerobic-anaerobic threshold, produces increased DNA damage in lymphocytes as detected by the single-cell gel electrophoresis (comet) assay (Hartmann *et al.*, 1994). This, along with certain deficiencies of natural antioxidants in the diet, could result in elevated oxidative mutagenesis (A:T to G:C) and higher accumulation of mutations (Qin and Huang, 1986; Gaziev *et al.*, 1995; Fraga *et al.*, 1996; Duthie *et al.*, 1996).

Compared to other subjects, cosmonauts exhibit high mutant frequencies, absence of G:C to A:T transitions at CpG sites, an overabundance of A:T to G:C transitions, and a small excess of G:C to T:A transversions and complex mutations. At the same time, the available information does not permit differentiation between the different factors to which cosmonauts are occupationally or environmentally exposed. The resolution of this question will require a larger study involving a general population drawn from the local environment as well as including trainees, and cosmonauts, before and after space flights. In addition, a larger number of mutants will have to be analysed at the molecular level.

CHAPTER IV. The Analysis of Mutations in T-lymphocytes of Trainees and Cosmonauts with Recent Long-Term Flight Experience

1. Abstract

Somatic mutations in five cosmonauts with recent long-term spaceflight experience and four age-matched trainees were analysed using the clonal *HPRT* assay. *Hprt* mutant frequencies in both cosmonaut and trainee groups were very similar, 17.2 ± 0.6 and $17.6 \pm 4.7 \times 10^{-6}$ respectively. These values are about 2-fold higher than the age-corrected values established for healthy, unexposed subjects in Western countries (Tates *et al.*, 1991; Branda *et al.*, 1993), and 2-fold lower than mutant frequencies observed in cosmonaut samples in our previous study (Khaidakov *et al.*, 1997). A total of 124 collected mutant clones were analysed using RT-PCR. Mutational spectra in studied groups were essentially similar. However, compared to the Western spontaneous mutational spectrum, they were significantly different ($p=0.031$ and 0.038), and exhibited a higher incidence of splice errors and complex mutations. At the same time, the pattern of base substitutions distribution did not differ in all compared datasets. Obtained data suggest that the space environment is not genotoxic at the *hprt* locus. Also, uniformly high *hprt* mutant frequencies observed in samples from individuals residing in Russia (Khaidakov *et al.*, 1997; Curry *et al.*, 1997; Jones *et al.*, 1995) are indicative of a higher mutagenic burden in Russia.

2. Introduction

Exposure to ionizing radiation in space is one of the major health concerns for long-term space missions. The doses received on the low Earth orbit are relatively small, ranging from 0.01 to 1.0 mGy per day (NCRP report, 1989). However, the quality of radiation in space, and the protracted and fluctuating manner of exposure, significantly complicates its biological effects, since a considerable fraction of space exposure involves high-LET radiation with a relative biological effectiveness (RBE) ranging from 1 to 60 (Hei *et al.*, 1988; Martin *et al.*, 1995). Moreover, high-LET radiation delivered in protracted manner is significantly more effective in causing oncogenic transformation when compared to the acute delivery of the same dose (Rossi and Kellerer, 1986).

The analysis of mortality among astronauts revealed an excess of cancer incidence significantly beyond expectations. In a survey conducted by Peterson *et al.* (1993) cancer was a registered cause of death in one astronaut out of total 20. Currently a total of 66 deaths have occurred among astronauts and cosmonauts. Twelve of them (3

astronauts and 9 cosmonauts) are attributed to diverse types of cancer (Zaselsky *et al.*, 1997).

This may be caused by several potentially mutagenic factors including ionizing radiation. Cytogenetic studies performed on the pre- and post-flight peripheral lymphocytes, post-flight samples demonstrate an excess of chromosomal aberrations after a 6-month period in space (Testard *et al.*, 1996; Yang *et al.*, 1997a, 1997b).

In our previous study, we analyzed mutations in samples from 5 cosmonauts (Khaidakov *et al.*, 1997). The *hprt* mutant frequencies in these samples were 2 to 5 times higher than in age-matched unexposed Western control, which may at least partially explain higher incidence of cancer among cosmonauts. The cosmonaut mutational spectra also showed distinct differences from the typical spontaneous mutational spectra observed in the Western *hprt* database suggestive of either environment and/or occupation-specific influences. Unfortunately, the available cosmonaut samples were not accompanied by matched controls. Therefore, an unambiguous conclusion as to the role of space flight could not be reached. Also, the time elapsed between the last space flight and the sampling time varied considerably, and in most cases was in excess of 2 years. This delay in sampling significantly reduces the likelihood of detecting fingerprints of the space flight related exposures (Akiyama *et al.*, 1992; da Cruz *et al.*, 1997).

To address these concerns we have collected samples from both trainees and cosmonauts with the recent long-term flight experience (Table 19). The purpose of the present study was to compare *hprt* mutant frequencies and mutational spectra derived from age-matched trainees and cosmonauts sharing similar environment, life style, and occupation.

3. Materials and Methods

Fresh blood samples were obtained in July 1997 from five cosmonauts who have completed long-term spaceflights, and four trainees (Table 19). Processing and analysis of samples was performed using methods and protocols as outlined in Chapter III .

4. Results

4.1 *Mutant frequencies*

Information on cosmonauts and trainees included in this study is presented in Table 19. The mean age in the cosmonaut and trainee groups was 40.4 ± 1.3 and 37.8 ± 2.4 years, respectively. Plating efficiencies in the samples ranged from 15.2% to 37.5%, while calculated mutant frequencies ranged from 7 to 30 x 10^{-6} with mean values for cosmonauts and trainees corresponding to 17.2 ± 0.6 and 17.6 ± 4.7 x 10^{-6}. Compared to unexposed Western subjects, the obtained averages are substantially higher (2-fold). At the same time, they are similar to the values of 18.5 ± 8.9 x 10^{-6} (Curry *et al.*, 1997[1]) reported for unexposed individuals residing in Russia. On the other hand, mutant frequencies in the new set of cosmonaut samples are about half the values we obtained earlier (Khaidakov *et al.*, 1997)

[1] Mean MF values for Russian twins (18.5 ± 8.9) were calculated from only those samples where plating efficiencies were equal or higher than 10%.

4.2 *Mutations and mutational spectra*

A total of 124 mutants selected from both cosmonaut and trainee samples were analyzed at the cDNA level. In four mutants no mutation could be found, and they were therefore not included in the mutational spectra. The cosmonaut mutant sequences are presented in the Table 20. Splice errors (40.7%), followed by base substitutions (32.2%), dominated the spectrum (Figure 7). Deletions, frameshifts, insertions and complex mutations comprised respectively 10.2%, 1.7%, 3.4% and 6.8%. Base substitutions were dominated by transitions (G:C → A:T 47.4% and A:T → G:C 15.8%), though transversions (G:C → T:A, 0%; G:C → C:G, 10.5%; A:T → T:A, 5.3%; A:T → C:G, 21%) were also recovered. In trainees (Figure 7) mis-splicing events and base substitutions constituted correspondingly 42.4% and 30.5%, whereas deletions, frameshifts, insertions and complex mutations comprised respectively 8.5%, 3.4%, 0% and 10.2%.

In both groups, 25% of all detected G:C → A:T transitions occurred at CpG sites, which is very similar to a ratio observed in spontaneous *hprt* database (Cariello *et al.*, 1996). A majority of the mis-splicing events involved exons 2 and 3. Apparently, the two deletions involving only a 5'-portion of the exons (mutants SP11-5 and SP11-69, trainees, Table 21), are also the result of a splicing error. The presence of a compulsory AG-dinucleotide at the 3'-prime end of deleted sequences suggests that legitimate acceptor splice site was inactivated and substituted by a similar downstream sequence.

A similar mechanism is likely involved in the majority of observed insertions as well. For example, in mutant SP17-22 (cosmonaut) a 14bp fragment from intron 8 adjacent to exon 9 was included in the coding sequence. The G:C → A:T substitution at

a TGG trinucleotide preceding inserted sequence could create a splice site competing with the legitimate splice site. Three mutants from two different trainees (SP11-15, SP11-30, SP12-5) had insertions between exons 8 and 9 of the same 42 bp fragment derived from intron 8. However, this fragment was intact in SP11-30, whereas in SP11-15 and SP12-5 the guanine in AG dinucleotide (pos. 41454) was substituted by cytosine and thymine, respectively. The nearest AG dinucleotide is located immediately upstream of this fragment. Another example of illegitimate splicing is mutant SP14-13 (trainee) carrying a 67 bp insert between exons 5 and 6. This insert is from the 5-prime portion of intron 5. In this case, the guanine in the required GT dinucleotide (pos.31636) is replaced by an adenine thus inactivating the legitimate donor splice site. The nearest cryptic splice site (GTAAGC) is located immediately downstream of inserted fragment.

Deletions were recovered in 8 and 6 mutants from cosmonaut and trainee datasets, respectively. Deletions recovered from cosmonauts were small and ranged from 4 to 28 bases, with half of them flanked by direct repeats 2-6 bp long. Two deletions in trainee samples were considerably larger and spanned 430 bp (exons 2-7, SP11-32) and 668 bp (SP12-1). Deletion in the latter case extended beyond the cDNA coding sequence. Three of six deletions were flanked by short direct repeats.

Complex mutations were found in 11 mutants. They exhibited different combinations of mutation events including two splicing errors (SP12-15, SP13-2), a splicing error and a base substitution (SP11-40, SP11-48, SP12-3, SP17-5, SP14-6), a splicing error and a deletion (SP10-1), a deletion and a insertion (SP16-11, SP18-8), or a frameshift and a base substitution (SP16-4).

4.3 *Comparison between cosmonauts' and trainees' datasets*

Distribution of mutations by class reveals that all three mutant collections, the current and earlier cosmonaut and trainee datasets, are rather similar (Fig.7). Monte Carlo tests performed on these datasets (Table 22) confirmed that these spectra are indeed similar ($p=0.59$ for 1st cosmonaut set *vs.* trainees, $p=0.80$ for the 2nd cosmonaut set *vs.* trainees, and 0.14 for two cosmonaut datasets). Similar values were obtained when the analysis was performed for the base substitutions mutational spectra (0.84, 0.32, and 0.20 respectively).

4.4 *Comparison with unexposed western male control*

Mutational spectra in all studied groups were significantly different from background control in western countries (Table 22). Due to the high similarity of mutational spectra in all cosmonaut and trainee groups, we also compared our pooled data with spontaneous mutational spectrum from *hprt* database. Monte-Carlo analysis of the mutation distribution by class revealed highly significant differences between these datasets ($p=0.0008$). This largely reflects the contribution of complex mutations and splice errors. A comparison of the base substitutions mutational spectra did not reveal any differences ($p=0.693$).

5. Discussion

The lack of induction of mutant frequencies, and the absence of any shifts in mutational spectra in the samples from cosmonauts compared to trainees, does not suggest the existence of any strong mutagenic influence of the space environment at the

hprt locus. At the same time, the data for both trainees and cosmonauts are considerably different from those obtained for the unexposed population in Western countries. The predicted MF for healthy unexposed individuals residing in Western countries at the age of 40 (and a CE of 25%) is in the range of 7.9 to 10.8 x 10^{-6} (Branda *et al.*, 1993; Tates *et al.*, 1991[2]). Our values are virtually identical in both Russian groups and about 2-fold higher than the age-corrected MFs in the Western control. This strongly suggests that the observed differences in the cosmonauts' samples are not related to the space missions, but rather are the consequence of the environmental or life-style factors. This view is further supported by the results obtained in other studies, where MFs determined in individuals residing in Russia also were considerably higher than in the age-matched Western controls (Jones *et al.*, 1995; Curry *et al.*, 1997).

In our previous analysis of the mutation in cosmonauts (Khaidakov *et al.*, 1997), the mean MF was more than 2-fold higher (38.0 ± 6.5) than in this current set (17.2 ± 0.6). Mean duration of space missions in second group was almost twice as long as in the original experiment (177.4 vs. 95.7 days). The original cosmonauts were older (47.4 years) compared to the current group (40.4 years), whereas their average plating efficiencies were quite similar (23.4 ± 1.9% *vs.* 25.6 ± 0.4%). This strongly indicates that observed differences could not be attributed to either space flight or a difference in cloning efficiency (Khaidakov and Glickman, 1996). We note, however, that despite being separated by only 7 years, the first group of cosmonauts belongs to an older and different generation with possibly different diet, life styles and pre- and post-flight medical exposures, which may at least partially contribute to our findings.

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[2] Calculation were performed for the age 40 years and CE at 25.6% using following formulas: ln MF = 3.25 - 0.52ln CE + 0.02age (Tates *et al.*, 1991), and ln MF = 1.99-1.13CE + 0.016age (Branda *et al.*, 1993)

Compared to the male spontaneous mutational spectrum from the *hprt* database, the pooled mutational spectrum from all cosmonauts' and trainees' samples was significantly different, and showed an excess of complex mutations, and splice errors (Figure 1). Complex mutants were represented by different classes of mutations. In the SP16-4 clone, one frameshift and one base substitution were detected. Base substitution occurred at the third base of the codon, and did not lead to a change of the amino acid. Mutant SP16-11 exhibited a combination of a small deletion located in exon 2 with the insertion of the second copy of exon 1 between exons 3 and 4. Among complex mutations involving combination of the aberrant splicing with other mutations, exons 2-3 skipping was implicated in all except one case. In addition, base substitutions accompanying exons 2-3 exclusions from cDNA resulted in mis-sense mutations in all such mutants. Exclusion of exons 2 and 3 comprises a large fraction of splice errors in *hprt* mutational spectra, and may range from 26% (Osterholm and Hou, 1998) to 51% (Khaidakov *et al.*, 1997). In many cases, no mutations could be found either in coding or flanking regions of involved exons. For example, in a study performed by Õsterholm and Hou (1998) on human lymphocytes, only 2 out of 14 independent mutants with exon 2-3 skipping had mutations in exons or adjacent intronic sequences. This suggests that splicing of exons 2 and 3 may be sensitive to a wide range of mutations. These mutations may not be necessarily positioned at the splice sites, and exert destabilizing (or excessively stabilizing) effect on the conformation of pre-mRNA, especially the stem-loop structure presumably harboring exon 2 splice site (Hennig *et al.*, 1995). In this context, this particular type of complex changes in cDNA sequence may be suspected as possible result of a single mutation leading to multiple consequences. The cDNA species

detected in mutant SP14-6 from the trainees' dataset seem to support this notion. This clone yields two cDNAs of different length. The normal length fragment contained small deletion in exon 9, whereas in the short fragment this deletion is complemented by the exclusion of exons 2 and 3.

Apparently, our findings present a potential contradiction with both the reported increase of frequency of chromosomal aberrations in the post-flight T-cells (Yang *et al.*, 1997; Testard *et al.*, 1997), and high contribution of cancer in cosmonauts mortality profile (Zaselsky *et al.* 1997). It can be explained by the specific ability of cytogenetic methods to detect large-scale rearrangements, which in majority of cases are incompatible with the cell viability. On the other hand, ability of *hprt* assay to recover deletions is limited to a maximum of 3.5 mb (Lippert *et al.* 1995).

It is also becoming increasingly clear that development of cancer may be initiated through the mechanism somewhat more sophisticated than the direct exposure to mutagens. There is evidence indicating that psychological stress is one of the life variables that may considerably increase risk of cancer (Watabe *et al.*, 1998). It is shown that the stress induces changes in balance of the wide array of hormones (Roozendaal *et al.*, 1997), activates transcription of several genes (Ueyama *et al.*, 1998; Autelitano, 1998), suppresses immunity (Derevenco, 1997), and inhibits apoptosis (Tomei *et al.*, 1990). Under these circumstances, a limited number of genes participating in signal transduction pathways or the cell cycle regulation could be a specific target for enhanced mutagenesis whereas the rest of the genome would remain relatively unaffected.

It is of some interest that the conclusions of a study on Russian twins performed in our laboratory (Curry *et al.*, 1997) differ from those presented here. The mutant

frequencies observed in the twins (30 ± 2 years of age), were even higher than in trainees and the second group of cosmonauts. This may reflect a lower average cloning efficiency (15.3 ± 1.7%) resulting in higher calculated mutant frequencies (Khaidakov and Glickman, 1996). Unlike the mutational spectra presented here, the distribution of mutations recovered in twins was rather similar to Western spontaneous control (p=0.296, Figure 3). In contrast, the distribution of the base substitutions was significantly different (p=0.02), which is precisely the opposite of what was observed in the 2nd cosmonauts' and trainees' datasets. However, the comparison between twins and the first set of cosmonauts' samples taken at the same time period revealed remarkable similarities (Figure 4), although shifts in cosmonaut base substitution mutational spectra were not as pronounced (p=0.229). These similarities, although insignificant, may indicate certain changes in environment, diet, or life style related mutagenic exposure in Russia in recent time.

The results obtained in both our previous and present studies of Russian cosmonauts indicate that changes in the *hprt* mutant frequencies and mutational spectra observed in the cosmonauts' samples are more likely region-specific than attributable to space flight factors. Consistently high mutant frequencies observed in all Russian samples suggest generally higher exposure to mutagens in this region. There are also some indications on a possible restructuring of mutagenic burden in post-transitional Russia.

Table 19. Description of samples obtained from cosmonauts and trainees

SAMPLE	AGE	FLIGHT YEAR	DURATION (DAYS)	CLONING EFFICIENCY	MUTANT FREQUENCY (x 10^{-6})
SP10	41 13.05.56	1992 1996/97	145 197	0.274	18.2
SP11	44 31.08.53	Trainee since 1991	–	0.308	30.1
SP12	39 21.06.58	Trainee since 1989	–	0.230	14.2
SP13	33 24.08.64	Trainee since 1991	–	0.340	7.7
SP14	35 21.04.62	Trainee since 1991	–	0.154	18.5
SP15	44 29.04.53	1995	140	0.255	15.4
SP16	40 09.10.57	1994 1996	184 197	0.227	18.3
SP17	36 06.02.61	1996	193	0.152	18.0
SP18	41 01.01.56	1992/93 1995/96	179 184	0.375	15.9

Table 20. *Hprt* cDNA mutations recovered in cosmonaut samples

Mutant	Mutation	Position	SEQUENCE CONTEXT	AA change
SP10-1	E2-3 loss and deletions	353-356 360-374	GATG <ATCT> CTC <AACTTT> AACTGGAAA	
SP10-2	E4 loss			
SP10-4	Deletion	69-72	TTTTG <CATA> CCTAA	
Sp10-6	G→A	143	TGAAC..A..TCTTG	Arg→His
SP10-7	E2-3 loss			
SP10-8	E2-8 loss			
SP10-10	E2-4 loss			
SP10-12	T→G	410	TATAA..G..TGACA	Ile→Ser
SP10-13	G→A	617	TGTTT..A..TGTCA	Cys→Tyr
SP10-14	E2-3 loss			
SP10-15	E2-3 loss and deletions	353-356 360-374	GATG <ATCT> CTC <AACTTT> AACTGGAAA	
SP15-1	E2-3 loss			
SP15-3	E2 loss			
SP15-4	A→T	403	GGAAG..T..TATAT	Asp→Val
SP15-7	E2-3 loss			
SP15-8	Duplication	300-301	TTTATC-AGACTG -AGACTGAAGAGCTATTG	
SP15-9	C→T	425	CAAAA..T..AATGC	Thr→Ile
SP15-10	E2-3 loss			
SP15-11	A→G	415	TTGAC..G..CTGGC	Thr→Ala
SP15-13	A→G	140	GACTG..G..ACGTC	Glu→Gly
SP16-1	E2-3 loss			
SP16-2	No mutation			
SP16-3	E2-6 loss			
SP16-4	G→A and Missing A	495 567	CTGGT..A..AAAAG GTTGT.. ..GGATA	Val→Val
SP16-5	E2-8 loss			
SP16-8	E7 loss			
SP16-9	T→G	104	AAGGG..G..GTTTA	Val→Gly
SP16-11	Deletion and insertion	33-37 E3/E4	ATTAG <TGATG> ATGAA E3-E1-E4	
SP16-12	E2-3 loss			
SP16-13	Missing A	567	GTTGT.. ..GGATA	
SP16-14	E2-3 loss			
SP16-15	E2-3 loss			
SP16-16	T→G	614	TCATG..G..TTGTG	Val→Gly
SP16-17	No mutation			
SP17-1	T→G	563	GTTTG..G..TGTAG	Val→Gly
SP17-3	Deletion	124-132	GACTA <ATTATGGAC> AGGAC	
SP17-4	G→A	570	TGTAG..A..ATATG	Gly→Gly
SP17-5	E2-3 loss and C→G	551	AATTC..G..AGACA	Pro→Arg
SP17-6	G→A	197	CCTCT..A..TGTGC	Cys→Tyr
SP17-8	E7 loss			
SP17-11	C→G	551	AATTC..G..AGACA	Cys→Tyr
SP17-15	E2-4 loss			
SP17-17	Deletion	153-156	GCTCG <AGAT> GTGAT	
SP17-19	G→A	143	TGAAC..A..TCTTG	Arg→His
SP17-20	Deletion	42-54	GATGA <ACCAGGTTATGAC> CTTGA	
SP17-21	G→C	119	TCATG..C..ACTAA	Gly→Ala

Table 20. Continued

Sp17-22[A]	Insertion 14 b	609-610	EX.8-ATTTTTTTTTTATAG-EX.9	
SP17-23[A]	Insertion 14 b	609-610	EX.8-ATTTTTTTTTTATAG-EX.9	
SP18-3	E2-6 loss			
SP18-4	A→G	545	ATTTG..G..AATTC	Glu→Gly
SP18-5	E2 loss			
SP18-6	E2-3 loss			
SP18-7	Deletion 6b	444-449	CTTTC <CTTGGT> CAGGC	
SP18-8	Deletion/ Insertion	208-236	TCAAG <GGGGG.. ..CTGCT> GGATT → TCAAG-CA-GGATT	
SP18-9	G→A	143	TGAAC..A..TCTTG	Arg→His
SP18-11	E2,3,8 loss			
SP18-12				
SP18-14	E2-3 loss			
SP18-16	C→T	610	TGAAT..T..ATGTT	His→Tyr
SP18-17	No mutation			
SP18-18	G→A	212	GGGGG..A..CTATA	Gly→Asp
SP18-20	E2-3 loss			

Notes: A – SP17-22, SP17-23 – insert from intron 8, 41441-41454, no mutations.

Table 21. *Hprt* cDNA mutations recovered in trainee samples.

Mutant	Mutation	Position	SEQUENCE CONTEXT	AA change
SP11-1	G→A	47	ACCAG..A..TTATG	Gly→Asp
SP11-3	E2-3 loss			
SP11-4	E2-6 loss			
SP11-5	E2 p.deletion	28-32	E1 <ATTAG> TGATG	
SP11-7	G→A	197	CCTCT..A..TGTGC	Cys→Tyr
SP11-8	Missing C	15-17	GCAGC.. ..CTGGC	
SP11-9	E4 loss			
SP11-10	A→T	133	TGGAC..T..GGACT	Arg→
SP11-14	G→C	143	TGAAC..C..TCTTG	Arg→Pro
SP11-15[A]	Insertion 42 b	609-610	Ex.8-aatatacttttaaatgtgaatttctggatttttttttatac-Ex.9	
SP11-16	Deletion	33-56	ATTAG <TGATG.. ..GACCT> TGATT	
SP11-17	E2-6 loss			
SP11-18	E7 loss			
SP11-20	Missing C	17	CAGCC.. ..TGGCG	
SP11-22	Deletion	327-330	GACCA <GTCA> ACAGG	
SP11-24	E2-8 loss			
*SP11-25				
SP11-27	A→G	415	TTGAC..G..CTGGC	Thr→Ala
SP11-28	G→A	212	GGGGG..A..CTATA	Gly→Asp
SP11-29	E2-3 loss			
SP11-30[B]	Insertion 42b		E8- aatatacttttaaatgtgaatttctggagttttttttatag -E9	
SP11-31	T→A	299	TTTTA..A..CAGAC	Ile→
SP11-32	Deletion	34-464	ATTAGT <GATGAT.. ..TAATCC> AAAGAT	
SP11-33	E5 loss			
SP11-34	E2 p. deletion	28-32	EX.1 <ATTAG> TGATG	
SP11-35	Missing T	318	TATTG.. ..	
Sp11-36	G→T	600	TTCAG..T..GATTT	Arg→ser
*SP11-37				
SP11-38	E6 loss			
SP11-40	E2-3 loss and A→G	401	TGTGG..G..AGATA	Glu→Gly
SP11-41	E8 loss			
SP11-42	G→A	212	GGGGG..A..CTATA	Gly→Asp
SP11-43	G→T	500	GAAAA..T..GACCC	Arg→Met
SP11-44	G→C	580	CCCTT..C..ACTAT	Asp→His
SP11-45	E2-8 loss			
SP11-48	E4 loss and A→G	451	TGGTC..G..GGCAG	Arg→Gly
SP11-49	E2-8 loss			
SP11-50	T→G	233	TGACC..G..GCTGG	Leu→CGG
SP11-53	E4 loss and A→G	451	TGGTC..G..GGCAG	Arg→Gly
SP11-55	A→G	530	GCCAG..G..CTTTG	Asp→Gly
SP11-56	E4 loss			
SP11-57	E2-3 loss			
SP11-59	E2-3 loss			
*SP11-61				
*SP11-62				
SP11-63	E2-3 or E2-8 loss			

Table 21. Continued

SP11-67	E2-3 loss			
SP11-69	E9 part.loss	610-626	E8- <CATGTTTGTGTCATTAG> TGAAA	
SP12-1	Deletion	98-766	TTTGG <AAAGG.. ..TTTGG> AAACAT	
SP12-2	G→T	58	ACCTT..T..ATTTA	Asp→Tyr
SP12-3	E2-3 loss And A→G	424	GCAAA..G..CAATG	Thr→Ala
SP12-5[C]	Insertion 42b	609-610	Ex.8-aatatacttttttaaatgtgaatttctggatttttttttatat-Ex.9	
SP12-10	E2-3 loss			
SP12-15	E2-3 loss E9 part loss	610-626	E8- <CATGTTTGTGTCATTAG> TGAAA	
SP13-2	E2-3,7 loss			
SP13-3	E2 loss			
SP13-4	Deletion	205-239	TGCTC <AAGGG.. ..CTGGA> TTACA	
SP13-5	C→T	151	TTGCT..T..GAGAT	Arg→Ter
SP13-7	No mutation			
SP14-1	C→T	113	TATTC..T..TCATG	Pro→Leu
SP14-3	G→A	617	TGTTT..A..TGTCA	Cys→Tyr
SP14-4	C→T	113	TATTC..T..TCATG	Pro→Leu
SP14-6	Deletion or deletion + E2-3 loss	636-642	ACTGG <AAAAGCA>AAATA	
SP14-9	A→T	413	AATTG..T..CACTG	Asp→Val
SP14-10	E2-3 loss			
***SP14-11**				
SP14-13[D]	Insertion 67b	E5-E6	E5-ataagttcacatttacttttaatataacatttatgactttctaacttagtat gcaccatcctaaag-E6	
SP14-15	No mutation			
SP14-16	No mutation			

Notes: A – SP11-15, insert 42 bp from intron 8, 41413-41454, G→C , pos. 41454.
B – SP11-30, insert 42 bp from intron 8, 41413-41454, no mutations
C – sp12-5, insert 42 bp from intron 8, 41413-41454, G→T, pos.41454
D – SP14-13, insert 67 bp from intron 5, 31636-31702, G→A, pos.31636

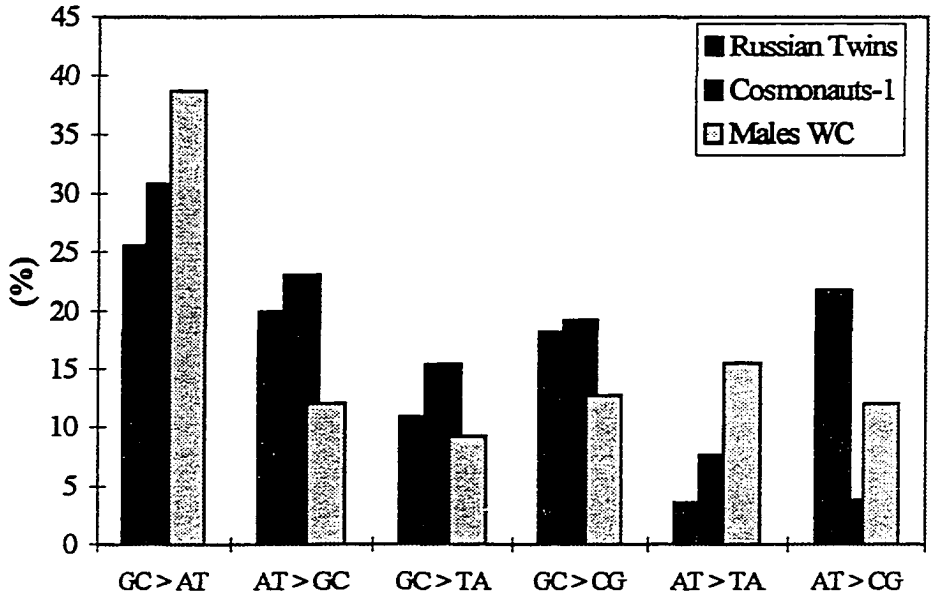

Figure 7. *Hprt* cDNA mutations in cosmonaut and trainee samples: Distribution by class. BS - base substitutions; SPL - splice errors; FRM - frameshifts; DEL - deletions; INS/DUP - insertions-duplications; COM - complex; TAN -tandem; WC – Western control;
Note: Comparisons between datasets have been performed using Monte Carlo test, and are summarized in Table 23;

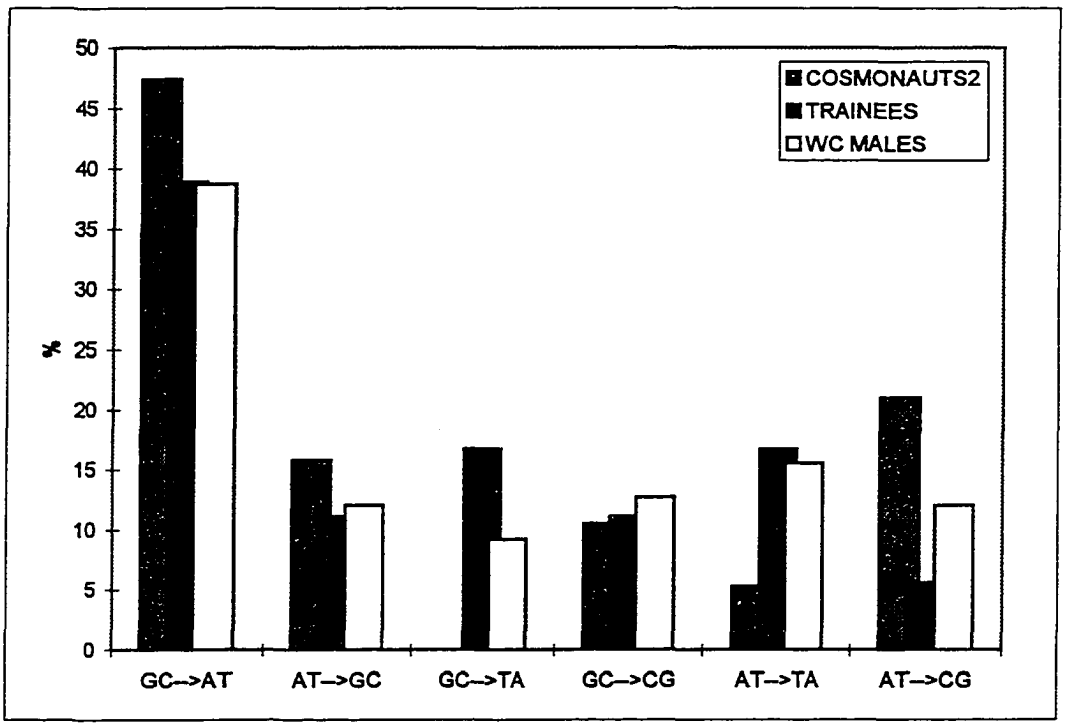

Figure 8. *Hprt* cDNA mutations in cosmonaut and trainee samples: Distribution of base substitutions.

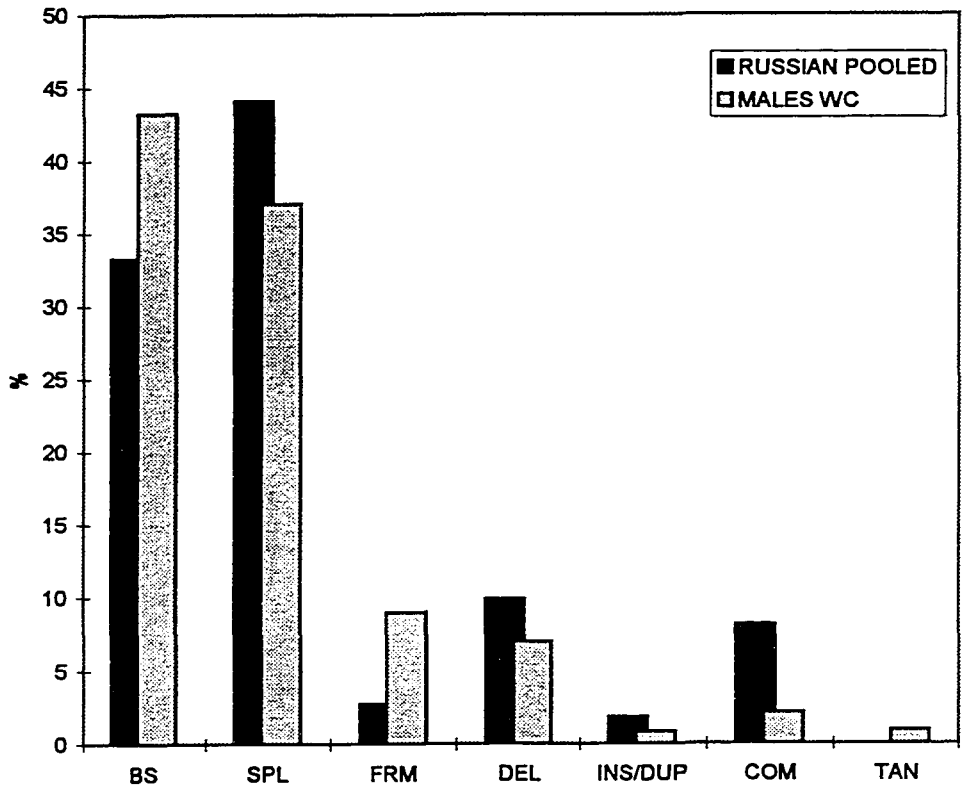

Figure 9. Comparison of pooled data from all cosmonaut and trainee samples With spontaneous data from *hprt* database (Cariello et al., 1996): Distribution by class.

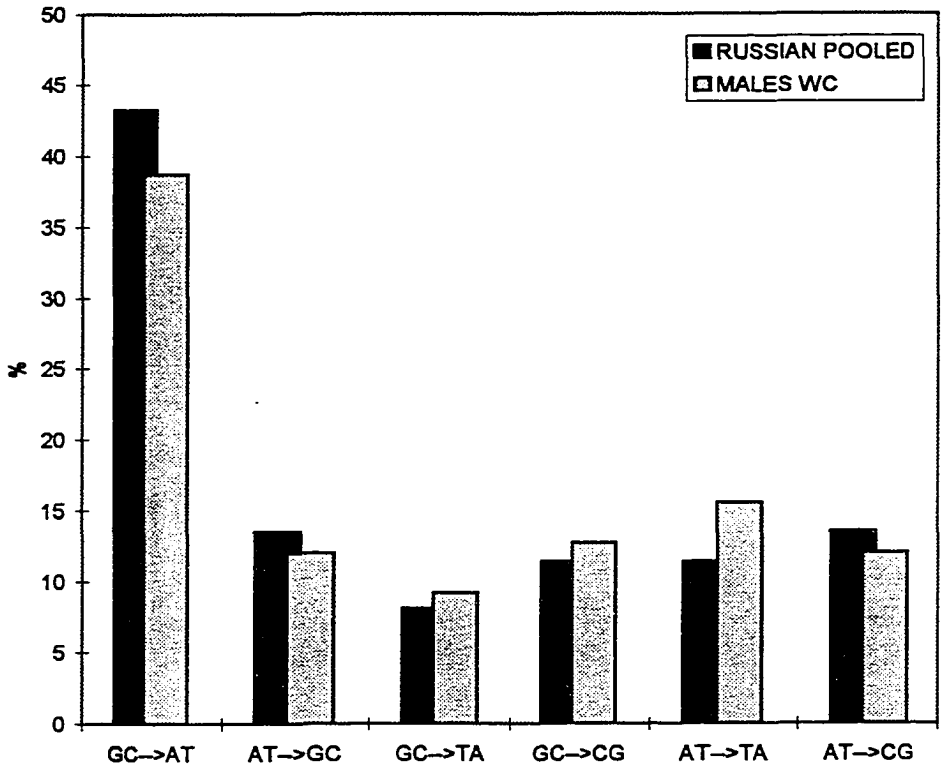

Figure 10. Comparison of pooled data from all cosmonaut and trainee samples With spontaneous data from *hprt* database (Cariello et al., 1996): Distribution base substitutions.

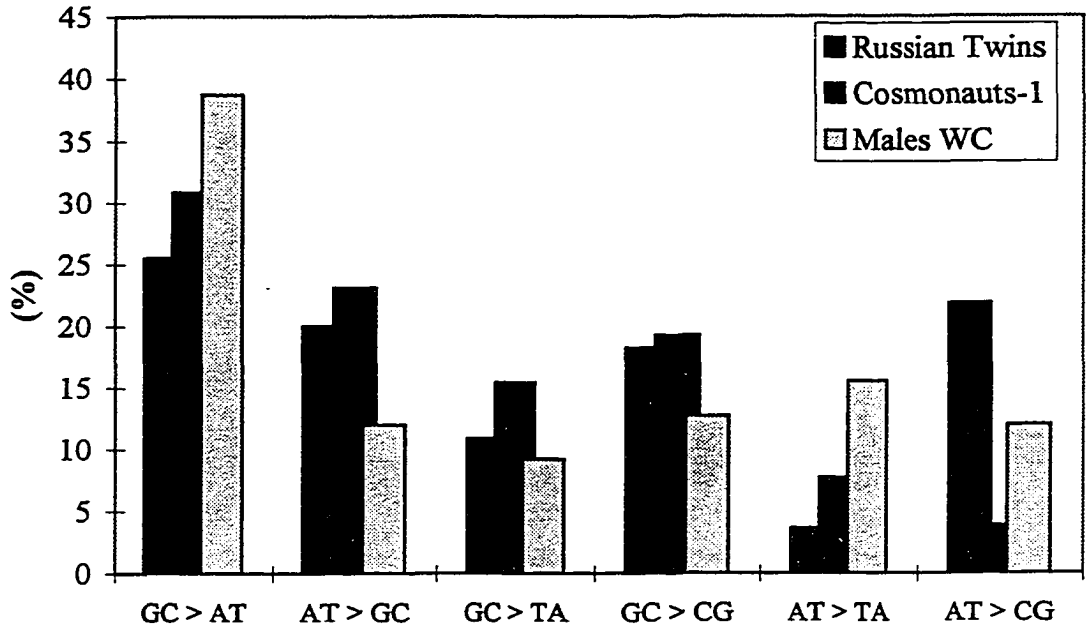

Figure 11. Distribution of base substitutions in the 1st Cosmonaut and Twin datasets.

Table 22. Monte Carlo test comparisons.

	COSM2	TRAINEES	MALES WC
DISTRIBUTION BY CLASS			
COSM1	0.141	0.586	**0.021**
COSM2		0.802	**0.031**
TRAINEES			**0.038**
POOLED			**0.0008**
DISTRIBUTION OF BASE SUBSTITUTIONS			
COSM1	0.203	0.844	0.229
COSM2		0.316	0.448
TRAINEES			0.908
POOLED			0.693

V. OVERALL DISCUSSION

Inverse Relationships between CE and MF in the *Hprt* Assay: Practical Implications

Inverse relationships between mutant frequencies and cloning efficiencies in the *hprt* assay represent a long-standing controversy (Albertini *et al.*, 1985). It has been speculated that inadequate conditions in the plating efficiency plates in some samples may contribute to exceedingly high apparent mutant frequencies (Cole *et al.*, 1988). Based on this assumption, all samples with a CE less than 10% were discarded to avoid a bias created by unusually high MFs observed in this group. Our assumptions on the mechanism underlying this phenomenon were based on the observation made by Hallahan and co-workers (1989) that medium from the cells subjected to cytotoxic levels of ionizing radiation acquires cytotoxic properties reversible upon addition of antibodies to Tumor Necrosis Factor α (TNFα). We speculated that presence of the 6TG in mutant selection plates could provoke a similar response in dying WT cells. Our scenario implied that under these conditions cells with higher initial viability would produce more cytokines and as a result make growth medium more cytotoxic for remaining 6TG resistant cells. Our findings confirmed the initial assumption demonstrating a consistent decrease in mutant plating efficiency with an increase of initial viability of WT clones in selective medium ($p < 0.05$, see Chapter II). These results suggest that in contrast to the commonly accepted model, mutant frequencies found in samples with low overall plating efficiencies reflect the actual values more accurately, and become significantly biased with increase of plating efficiency.

Our findings also indicate that mutagenicity of agents producing a relatively weak mutagenic response cannot be adequately assessed without correction for their cell killing potential. This is because cytotoxicity *per se* can cause a comparable increase in MFs via its suppressing effect on the viability of mutant cells. Moreover, the mutational spectra derived from a cytotoxicity-mediated increase of mutant frequencies should not be any different from spontaneous mutational spectrum due to the non-selective nature of improved recovery of mutants.

Effects of the Space Environment

Compared to Western background control, samples from the first set of cosmonaut samples displayed highly elevated *hprt* mutant frequencies and significant shifts in mutational spectra. These changes could be attributed both to the effects of ionizing radiation exposure in space, other job-related factors, or certain environmental or tobacco mutagens (Mazur and Glickman, 1987; Wu *et al.*, 1995; Tiano *et al.*, 1994; Dizdaroglu, 1985; see discussion in Chapter III). The lack of age-matched controls precluded more definite conclusions, although studies on unexposed Russian residents (Jones *et al.*, 1995) and Russian twins (Curry *et al.*, 1997) also found elevated MFs, suggestive of either genetically determined or environmental contributing factors. A comparison of the distribution of mutations by class in the *hprt* database and both Russian datasets did not demonstrate striking differences except for moderate relative abundance of deletions and complex mutations in cosmonauts' and Western spontaneous mutational spectra. However, a more detailed analysis of mutation distribution within the base substitution class reveals peculiar similarities in both Russian datasets, showing a virtually identical

degree of excess of A:T>G:C and lack of G:C.A:T transversions (Fig. 11). Taken together, the available data were more suggestive of a region-specific than occupation-specific cause of observed changes in cosmonauts' MFs and mutational spectra.

Analysis of the second cosmonaut and age-matched trainee sets revealed similar trends in both MFs and mutational spectra. The mean MFs were virtually identical in both groups (17.2 vs. 17.6 x 10^{-6}). Compared to trainees, cosmonaut mutational spectrum did not show any notable deviations as well (Figs.7 and 8). At the same time, all three datasets differed significantly from the Western background control (Table 23).

Combined data from two studies confirm our preliminary conclusion that the space environment is not genotoxic at the *hprt* locus, and elevated MFs are characteristic for the Russian residents in general. Lack of the post-flight *hprt* mutant induction is a seeming contradiction with the reported higher incidence of cancer-related deaths in this occupational group (Zaselsky *et al.*, 1997). One explanation is that neoplastic processes in this particular case were not initiated by a global increase in mutagenesis, but rather by a combination of epigenetic factors including immunosuppresion (Derevenco, 1997) and inhibition of apoptosis (Tomei *et al.*, 1990).

Our findings emphasize the importance of the issue of comparability between datasets from ethnically, socially, regionally or even culturally different groups. Lifestyle, dietary habits, typical mixture of pollutants in the countries of residence, levels of genetic heterogeneity, levels of social acceptance of smoking, *etc.*, create a unique environment for mutagenesis. Without an adequate background control from the same group comparison of obtained data with datasets derived from individuals residing in Western countries can lead to erroneous conclusions. For example, in a study performed on a

group of children from Chernobyl (Dubrova *et al.*, 1996), the authors analyzed frequency of mutations in minisatellite loci in T-lymphocytes. A group of children from England served as a reference, and results indicated that mutation frequency in samples from Chernobyl were approximately two-fold higher, which justified the authors' conclusion regarding effects caused by ionizing radiation exposure. However, according to data from Jones *et al.* (1995) and our laboratory (Curry *et al.*, 1997), background *hprt* mutant frequencies in an unexposed Russian population can be 2-3-fold higher than in a Western control, a fact that seriously undermines the validity of their findings. Interestingly, a similar study performed on cleanup workers from Chernobyl compared with samples from individuals residing in Russia (*i.e.* sharing the same lifestyle, similar genetic makeup, and environment) did not reveal any significant differences in chromosome translocations, as well as in *hprt* and GPA mutant frequencies (Moore *et al.*, 1997).

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VII. APPENDIX

Mutagenicity and Mutational Specificity of Etoposide

1. Introduction

Etoposide is a derivative of podophylotoxin, a natural compound, successfully used for treatment of a variety of cancers (Sinha, 1995, section 2.3). Podophylotoxins are found to be potent inhibitors of topoisomerase II, the enzyme ubiquitous in all cell types, which is responsible for relieving torsional stress in supercoiled DNA during replication. Similar to ionizing radiation, the majority of inhibitors of topoisomerase II introduce double-strand breaks in mammalian DNA (Osheroff, 1989), which in turn result in the formation of deletions of various sizes (Han *et al.*, 1993; Berger *et al.*, 1991). An elevated incidence of secondary leukemias with a specific type of translocation has been found in patients receiving chemotherapy with inhibitors of topoisomerase II (Kobayashi *et al.*, 1997; Felice *et al.*, 1998). This suggests that similar mutational spectra can be expected from human cells. In several *in vitro* experiments it has been shown that etoposide induces a significant mutagenic response in the *hprt* gene of CHO cells (Singh and Gupta, 1983a, 1983b; Pommier *et al.*, 1985; see section 2.6).

The purpose of this study was: i) to analyse the sensitivity of *hprt* target in human cells to treatment with a clastogen, both in terms of mutagenic response and mutational spectrum; and, ii) to compare magnitude and quality of mutagenic response in normal human T-lymphocytes and immortal lymphoblastoid (TK6) cells.

2. Background: Topoisomerase II Inhibitor - Etoposide

2.1 Structure and function of topoisomerase II

Many processes in DNA require certain topological changes, which are partially mediated by DNA topoisomerases. In eucaryotic organisms two types of topoisomerases are found. They introduce transient single- (type I) or double- (type II) strand breaks in DNA thus relieving its supercoiling. Both topoisomerases have considerable overlap in their functions. However, only type II topoisomerase is vital for the survival of the cells (Holm *et al.*, 1985), which is linked to the unique decatenating ability of topoisomerase II necessary for the disjunction of chromatids in mitosis (Dinardo *et al.*, 1984) and meiosis (Rose *et al.*, 1990).

In eucaryotes, topoisomerase II is a major component of the matrix or scaffold associated regions (MARs or SARs) which are AT-rich regions several hundred base pairs in length. MARs are responsible for chromatin loop attachment to the nuclear scaffold, and contain multiple topoisomerase II recognition sites (Adachi *et al.*, 1989; Blasquez *et al.*, 1989; Razin *et al.*, 1991). In the mammalian genome topoisomerase preferentially binds to MARs (Sperry *et al.*, 1989), which indicates that this enzyme is involved in chromosome structure.

Unlike yeast and drosophila, human cells carry two genetically distinct isoforms of type II topoisomerase, α and β, which map to 17q21-22 and 3p24, respectively (Tan *et al.*, 1992; Jenkins *et al.*, 1992). These isoforms are expressed differently, topoisomerase II β is maintained at a constant level throughout the cell cycle whereas the topoisomerase II α levels change in a cell-cycle specific manner (Hwang *et al.*, 1989; Woessner *et al.*,

1991; Kimura *et al.,* 1994). The amount of topoisomerase II α is lowest in G1, where the half-life of the enzyme is several-fold shorter than in asynchronous population. This is likely coupled with the cycle of chromosome condensation/ decondensation (Heck *et al.,* 1988). Isoforms α and β share only 68% homology (Austin *et al.,* 1993), but they are functionally very similar (Austin *et al.,* 1995; Cornarotti *et al.,* 1996), although they apparently have different locations in the nucleus (Capranico *et al.,* 1992).

The activity of the topoisomerase II is directly related to phosphorylation of several sites within its C-terminus. The treatment of the enzyme with protein kinase results in several-fold increase in its activity, whereas its dephosphorylation by alkaline phosphatase abolishes it almost completely (Saijo *et al.,* 1990: Wells *et al.,* 1995). Phosphorylation of topoisomerase II is cell-cycle specific, and shows maximum in G2-M phases of the cell cycle (Heck *et al.,* 1989; Saijo *et al.,* 1992; Well and Hickson, 1995).

It is now established that type II topoisomerase can catalyse double-strand breaks in DNA to form a DNA gate for the passage of another double-stranded segment (Hsieh, 1990). Until recently, there were two models coexisting that explain mechanics of the DNA transportation process in the dimeric enzyme molecule. In the one-gate model DNA segment to be transported (T segment) passes through the DNA gate and is docked in the cavity. Then cleaved DNA segment (G) is religated and dissociates from one of the subunits of enzyme allowing T segment to exit without crossing G segment second time. In the two-gate model, T segment passes through the entire interfacial channel between two subunits, and exits through the second gate on the other side of the enzyme.

Both models were recently tested in the experiments based on the fact that topoisomerase II acts as an ATP-dependent clamp, closing upon binding of ATP and

opening again after ATP hydrolysis (Roca and Wang, 1992, 1994). If the one-gate model is correct, the DNA ring containing the T segment should be irreversibly trapped in the clamp when non-hydrolysable ATP analogue is used in a single step decatenation. However, the DNA ring carrying the G segment remained bound to the enzyme, whereas the DNA ring with the T segment was found free in solution. This finding favours the two-gate model.

The three-dimensional structure of human type II topoisomerase has been recently determined (Schultz *et al.*, 1996). There are three domains, a globular core domain ~ 90Å, which is, flanked by two smaller domains approximately 50-60 Å in diameter. The core domain has a two-fold symmetry suggesting a homodimeric structure. Upon the binding of a non-hydrolysable ATP analogue topoisomerase displays an interesting conformational change yielding only two-domain (90Å and 60 Å) image which is consistent with the "clamp" model.

The enzyme functions as homodimer (Tennyson and Lindsley, 1997), although it is not necessarily the only functional form of topoisomerase II. It has been shown that in human cells a considerable fraction of enzyme molecules may exist as heterodimers composed of α and β isoforms (Biersack *et al.*, 1996) without losing enzymatic activity. In addition, topoisomerase II is capable of binding to other nuclear proteins, termed Topoisomerase II-interactive proteins, TIPs (Kroll, 1997) in the region partially overlapping domain responsible for homodimerization. It is also of interest that both topoisomerase II isoforms were found to bind p53 *in vitro* (Yuwen *et al.*, 1997), and thus probably interfere with the regulation of the cell cycle.

Structurally, topoisomerase II is composed of three domains including highly conserved N-terminal region, core region and highly variable C-terminal region. N-terminal fragment is homologous to ATP-binding subunit B of bacterial gyrase, whereas central part is similar to subunit A responsible for catalytic function of the bacterial enzyme (Reece and Maxwell, 1991). Analysis of the crystal structure of the yeast enzyme (Berger *et al.*, 1996) revealed presence of DNA-binding motif in a core region able to protect ~16 base pairs of duplex, which agrees with other experimental data (see section 2.2).

On the primary DNA sequence level, topoisomerases II from different species have several conserved regions separated by non-conserved sequences of different lengths (Jensen *et al.*, 1996). A deletion of any conserved region results in loss of enzyme function. Interestingly, the variable C-terminal region which does not have an equivalent counterpart in bacterial gyrase (Shiozaki and Yanagida, 1991) is also essential in eucaryotes as deletions exceeding a certain size render a non-viable phenotype, even though catalytic function remains intact (Jensen *et al.*, 1996). Truncation further than position 1352 affects nuclear localisation of the enzyme indicating that some proximal parts of C-terminal region direct transport of topoisomerase II within nucleus (Adachi *et al.*, 1997).

2.2 Topoisomerase II Binding and DNA Sequence Specificity

Each enzyme molecule strongly protects 22-25 nucleotides of DNA (Thomsen *et al.*, 1990; Lee *et al.*, 1989). The minimal length of DNA substrate required for the

optimal cleavage is in the range of 28-mer or greater (Thomsen *et al.*, 1990), although in one study, a 16-mer was found to be sufficient for cleavage (Lund *et al.*, 1989). Later studies by the same group using TIMBER (Triplex Interference Mapping by Binding Element Replacement) revealed that core binding sequence spans 10 bp region from -3 to +7 position relative to the cleavage site (Spitzner *et al.*, 1995).

The cleavage of naked DNA by topoisomerase II involves two asymmetric single-strand cleavages separated by four bases. Depending on the strength of the recognition sequence, cleavage may involve either two or just one strand. Strong consensus sequences exhibiting dyad symmetry in most cases yields double-strand breaks (Muller *et al.*, 1988). The definition of the topoisomerase II consensus sequence has undergone considerable change over the last 10 years (Table 23). Data from different laboratories derived from *in vitro* studies indicate the topoisomerase II consensus sequence to be quite degenerate.

In vivo activity of topoisomerase is restricted by accessibility of potential cleavage sites determined by chromatin structure (Udvardy and Schedl, 1991; Capranico *et al.*, 1990). As a result, cleavage pattern in naked DNA is very much different from what is observed *in vivo*. Cleavage *in vivo* occurs at sites that are secondary in naked DNA, and are confined to linker DNA sequences. DNA regions with fixed nucleosome positioning are resistant to topoisomerase II action whereas linker DNA regions are efficiently cleaved. These findings are further confirmed by an *in vitro* study, where the

Table 23 Topoisomerase II consensus sequence and local sequence preferences for Topoisomerase II inhibitors

Topoisomerase II origin	Consensus sequence -987654321▼123456789	Author
Drosophila	ATGAGGATGACGATG▼AGCGCATTGTTAGATG	Sander and Hsie, 1983
Drosophila	GtnA/TAY▼ATTnATnnG	Sander and Hsie, 1985
Chicken	RnYnnCnnGY▼nGKTnYnY	Spitzner and Muller, 1988
Mouse ND Mouse VM-26 Mouse AMSA	noA▼----noT acgaaC▼acccGttcgt gaat▼Agctatac	Pommier *et al.*, 1991
Drosophila Chicken, VM26 Mouse, VM26 Mouse, AMSA	TAn▼nTAnnTA nnC▼nnnnnYn nnC▼nnnnnnn T/ARn▼AnnTnnT/A (Pur▼Pir)n	Spitzner *et al.*, 1994
Phage T4	A/CAGRTn▼GTTAAAC	Freudenreich & Kreuzer, 1993

ND - analysis has been performed in absence of topoisomerase II inhibitors.

addition of histone H1 to naked DNA led to a decrease in the relaxation activity of topoisomerase II (Galande and Muniaappa, 1997).

There is also some evidence suggesting that topoisomerase II exhibits higher affinity to certain DNA conformations and secondary structures and in some cases may be relatively indifferent to a sequence context. It has been observed that supercoiled DNA presents a more efficient target for topoisomerase II. Moreover, the enzyme cleavage sites are clustered in close proximity to the hairpin structures (Pognan and Paoletti, 1992). In another study, the enzyme bound more readily to minicircle preparations with insert assuming Z conformation than to minicircles without inserts (Glikin *et al.*, 1991). It should be noted, however, that in a similar experiment (Choi *et al.*, 1995) , the degree of supercoiling strongly affected efficiency of DNA cleavage by topoisomerase II, although sequence specificity remained unchanged. The curvature of DNA fragments seems to be one of the determinants of topoisomerase II binding. Affinity for the α isoform is the highest for curved DNA and linear Z-DNA, is somewhat lower for supercoiled DNA, and considerably lower for the linear B-DNA (Bechert *et al.*, 1994). Hairpin structures were found to be cleaved by topoisomerase II at spatially fixed position (3'-base of the stem) regardless of sequence context (Froelich-Ammon *et al.*, 1994). DNA scission required a 3'- double-stranded/single-stranded junction with at least 8 bp single or double-stranded tail on the 5'-end, whereas the elimination of the hairpin and the formation of double-stranded template from the same oligomer completely abolished cleavage.

Topoisomerase II sequence specificity may be further compromised by the certain types of spontaneous DNA lesions acting as potent stimulators of topoisomerase II activity. It has been found that abasic sites which can be a result of either oxidative

damage or exposure to different mutagens (ionizing radiation, alkylating agents etc.) dramatically increase forward rate of topoisomerase II-mediated DNA cleavage (Kingma *et al.*, 1995,1997). In terms of relative concentrations producing similar effect abasic sites were about 2000 times more effective than etoposide. This property along with the fact that distribution of spontaneous or mutagen-induced abasic sites within genome appears to be non-random and chromatin structure-dependent (Legault *et al.*, 1997) could affect apparent sequence specificity of the enzyme.

2.3 Inhibitors of Topoisomerase II: Clinical Applications and Adverse Effects

Inhibitors of topoisomerase II represent one of the most efficient classes of chemotherapy agents used for treatment of lung cancer, breast cancer, leukemias and lymphomas, and many other neoplasias (Sinha, 1995; Isaacs *et al.*, 1995). However, it has been observed that treatment regiments incorporating inhibitors of topoisomerase II result in an increase in secondary malignancies (see for reviews Ratain and Rowley, 1992; Anderson and Berger, 1994; Smith *et al.*, 1994; van Leeuwen, 1996; Zhang *et al.*, 1996; Pedersen- Bjergaard *et al.*, 1997; Kobayashi *et al.*, 1997; Felice *et al.*, 1998). Most of the secondary malignancies were accompanied by rearrangements involving the MLL (ALL-1) gene at 11q23. The validity of these findings is additionally strengthened by *in vitro* data on different types of cells where exposure to inhibitors of topoisomerase II reproducibly resulted in DNA cleavage in a specific site located within MLL breakpoint cluster region (Aplan *et al.*, 1996; Stanulla *et al.*, 1997).

A fundamental determinant of chemotherapy efficacy in cancer treatment is to cause more damage to malignant rather than normal cells. Unlike normal cells, tumour

cells acquire varying degrees of independence from the normal internal mechanisms that regulate proliferation. As a result, tumours usually exhibit higher mitotic activity than surrounding normal cells (Tsujihashi *et al.*, 1989).

Cytotoxicity of inhibitors of topoisomerase II at least partially follows a pattern of the cell cycle-specific changes in topoisomerase II activity, which in itself serves as one of the markers of proliferation (see section 2.5). In non-dividing mammalian cells, concentration of topoisomerase II is very low (Sullivan *et al.*, 1987a, 1987b; Heck *et al.*, 1988; Dimanche-Boitrel *et al.*, 1994), whereas in proliferating cells, and especially in G2-M phase its concentration increases several-fold (Heck *et al.*, 1988).

Several types of transformed and tumour cells exhibit higher levels of topoisomerase II activity (van der Zee *et al.*, 1991; Fry *et al.*, 1991; Cornarotti *et al.*, 1996) which in some cases may become independent from the cell cycle (Hsiang *et al.*, 1988). Moreover, there is a negative regulation loop between p53 and topoisomerase II. Topoisomerase II is able to bind wild type p53 (Yuwen *et al.*, 1997) thus preventing p53 from performing its regulatory function. At the same time, wild type p53 downregulates topoisomerase II expression by binding to a promoter region (Sandri *et al.*, 1996). However, mutant forms of this pro-oncogen fail to do so which is probably an important component of cancer pathogenesis.

2.4 Mechanism of action

There is a remarkable difference in the mechanism of action of the various agents targeting topoisomerase II. Inhibitors may affect specifically DNA binding, DNA cleavage, or religation. For example, the inhibitor Ro 15-0216 has no effect on religation,

but greatly stimulates DNA cleavage step (Sorensen *et al.*, 1992). In contrast, topoisomerase II inhibitors anthracycline and aclarubicin suppress the initial non-covalent DNA binding reaction, and prevents DNA breakage.

The characterisation of etoposide-induced changes in the function of topoisomerase II was studied using cleavage/religation reaction of *Drosophila melanogaster* topoisomerase II (Osheroff, 1989). Etoposide at concentrations comparable to those used in chemotherapy shifted cleavage-religation equilibrium in favour of cleavage. As a result of exposure to etoposide, formation of double and single-strand breaks was enhanced respectively 5-6 and 4-fold. Using a technique allowing uncoupling enzyme's forward cleavage reaction from reverse religation reaction by trapping cleavable complex in presence of calcium (Osheroff and Zechiedrich, 1987), etoposide was shown to inhibit religation reaction 3-fold. Similar results were obtained for another topoisomerase II inhibitor, amsacrine (Robinson and Osheroff, 1990).

The induction of topoisomerase II-mediated DNA cleavage by etoposide depends to a substantial degree on the presence of 4'-OH in the molecule. At least cytotoxicity, as well as intensity of DNA cleavage are not induced by etoposide derivatives lacking this group (Sinha *et al.*, 1990). Etoposide binds to the same interaction domain on enzyme molecule as the diverse group of other inhibitors of topoisomerase II (Corbett *et al.*, 1993). In non-turnover DNA catenation assays, several inhibitors were tested of which only etoposide was unable to inhibit the DNA strand passage step. In competition experiments, etoposide reversed the inhibition of strand passage by genistein, CP-115, 953 and amsacrine, but failed to do so in the presence of novobiocin. Similarly suggestive results were obtained in another study (Huff and Kreuzer, 1990), where T4 topoisomerase

carrying mutation causing resistance to α-AMSA had altered sensitivity to an array of different topoisomerase II inhibitors.

The binding of inhibitors to topoisomerase II leads to formation of cleavage complexes. These complexes may have unique preferences for certain consensus sequences depending on the inhibitor employed (Pommier *et al.*, 1991; see section 2.2). It has been found that etoposide induces topoisomerase II mediated DNA cleavage preferentially at sequences with a cytosine residue immediately 5'- to the cut (Pommier *et al.*, 1991; Burden *et al.*, 1996). The analysis of 22 cleavage sites in a 564-bp fragment of pBR322 plasmid (Burden *et al.*, 1996) revealed that C_{max} values (maximal cleavage induced by etoposide) varied ~60-fold, and that there was no correlation between C_{max} changes and levels of etoposide affinity to DNA in the sites under study. The inhibition of religation in the presence of etoposide varied ~30-fold among sites, and these variations showed a strong negative correlation with C_{max} values ($r = 0.86$). This suggests that etoposide inhibits religation in a sequence-specific manner.

2.5 Cytotoxicity of etoposide

Etoposide produces both single- and double-strand breaks in DNA (Osheroff, 1989). However, only double-strand breaks and sister chromatid exchanges show a correlation with the cytotoxic action of etoposide (Long and Stringfellow, 1988; Noviello *et al.*, 1994). The formation of cleavable complexes and chromosome-type aberrations are causally associated, and there is a strong positive correlation between them as well (Suzuki *et al.*, 1995).

Cytotoxicity of topoisomerase II inhibitors is consistently correlated to the cellular activity of the enzyme. Cell lines with lower RNA content and topoisomerase II activity exhibit markedly reduced chemosensitivity accompanied by a greatly diminished clastogenic efficiency of topoisomerase II inhibitors (Hong, 1989). Accordingly, the overproduction of topoisomerase II confers increased sensitivity, as has been shown in SV-transformed cell lines derived from normal, AT, and XP donors with abnormally high levels of topoisomerase II (Smith and Makinson, 1989). Enzyme activity may also serve as determinant of potential efficiency of chemotherapeutic agents. Comparison of generally resistant bladder carcinoma cells with more sensitive testis tumours revealed that their difference in response to amsacrine, adriamycin and etoposide correlates with topoisomerase II content and induced DNA-breakage (Fry *et al.*, 1991). Also, the transfection of drug-resistant tumour cells carrying mutation in the topoisomerase II alpha gene with the wild-type copy restores sensitivity to inhibitors (Asano *et al.*, 1996).

Inhibitors' cytotoxicity does not always coincide with peaks of enzyme activity, or DSBs, which indicates on superimposition of different mechanisms of cell killing. For example, in a study conducted by Chow and Ross (1987) it has been found that etoposide-induced DSBs reached maximum during G2-M phase, whereas maximum cytotoxicity was timed to S phase. In synchronised HeLa cells m-AMSA and etoposide produced highest cleavage in mitosis (4-15 fold *vs.* S phase) which was not paralleled by increase in topoisomerase II activity (Estey *et al.*, 1987).

The lack of complete chromosome condensation and non-disjunction of sister chromatids are well-known consequences of topoisomerase II poisoning or topoisomerase II deficiency (Wood and Earshaw, 1990; Adachi *et al.*, 1991; Downes *et*

al., 1991;Warburton and Earnshaw, 1997). Topoisomerase II-depleted mitotic extracts from Xenopus eggs do not prevent topoisomerase II-rich HeLa nuclei from conversion to mitotic chromosomes (Adachi *et al.*, 1991). On the contrary, chicken erythrocytes nuclei with low enzyme content fail to condense chromosomes. These functions of topoisomerase II seem to be independent from its ability to produce DSBs. This follows from the studies where topoisomerase II poisoning with inhibitor ICRF-193 that produces neither topoisomerase II-DNA complexes nor DSBs, but nevertheless prevents anaphase segregation in HeLa and P*tk2* cells (Clarke *et al.*, 1993). Inhibition of enzyme by ICRF-193 appears to affect the conversion of 300 nm diameter chromatin fibres to 600 nm chromatids (Ishida *et al.*, 1994). Despite this, cells proceed through mitosis in the absence of chromosome segregation, become polyploid and lose viability. Equally suggestive results were obtained by Anderson and Roberge (1996) in BHK and tsBH2 cells. In their study, all inhibitors tested, including etoposide and ICRF-187, caused G2 arrest. Cells treated with etoposide showed chromosomal fragmentation, whereas those from ICRF-187-treated cells were elongated and entangled.

Recently it was suggested that the cytotoxicity of some topoisomerase II inhibitors may be at least partially caused by a collision of progressing replication forks with stabilised cleavable complexes (Zhang *et al.*, 1990). In experiments with human CCRF-CEM leukaemia cells (Qui *et al.*, 1996), Topoisomerase II α isozyme formed complexes with nascent DNA in cells exposed to VM-26, whereas the β isozyme was bound to bulk DNA. In VM-26 sensitive cells, teniposide enhanced topoisomerase II α to nascent DNA 5-fold compared to bulk DNA. However, in VM-26-resistant cells, equitoxic treatment resulted in opposite ratio, suggesting that Topoisomerase II alpha is

involved in DNA replication. Further studies (Catapano *et al.*, 1997) revealed that cytotoxicity of VM-26 correlated with the number of drug-stabilised cleavable complexes, and the severity of DNA chain elongation inhibition. The analysis of replication fork progression from the putative origin of replication positioned upstream of the topoisomerase II cleavage site located in the first exon of c-myc gene showed that in VM-26 treated cells replication forks were specifically arrested at the topoisomerase II DNA cleavage site.

Programmed cell death or apoptosis was discovered relatively recently (Kerr *et al.*, 1972). It is a universal mechanism playing a fundamental role in embryonic development, tissue maintenance, and strategy employed by the cell populations in order to cope with adverse environmental conditions including exposure to mutagens. It is a multilevel reaction involving *de novo* RNA and protein synthesis and the activation of several genes (Wyllie, 1995). Inhibitors of topoisomerase II are known to trigger apoptosis in different types of normal and tumour cells including fibroblasts (Mizumoto *et al.*, 1994), lymphocytes (Roy *et al.*, 1992), neurones (Nakajima *et al.*, 1994), thymocytes (Walker *et al.*, 1991; Bruno *et al.*, 1992; Onishi *et al.*, 1994), epithelial cells (Fukuda *et al.*, 1996). With the inhibitors promoting stabilisation of the cleavable complex, this process is characterised by two waves of DNA cleavage: the first being caused by formation of inhibitor-topoisomerase II-DNA complexes, and the second caused by endonuclease activity (Walker *et al.*, 1991). Since DSBs and SCEs are strongly correlated with cytotoxic action of inhibitors (Pommier *et al.*, 1985; 1988) the choice of apoptotic pathway seemed to be similar to that of other clastogens, such as ionizing radiation. However, recent studies revealed that formation of DSBs is not a primary

condition for the initiation of apoptosis in the drug-treated cells. The employment of inhibitors lacking the ability to stabilise cleavable complexes and induce DSBs (ICRF-154, MCT-16) results in a pronounced apoptotic reaction as well (Onishi *et al.*, 1994). Inhibitor-induced apoptosis exhibits certain selectivity with regard to the phase of the cell cycle. It has been reported that in experiment with rat thymocytes subjected to teniposide, a selective loss of G_0 cells was observed with no apparent changes in other fractions in the cell cultures (Bruno *et al.*, 1992). However, these findings are contradicted by the results from a similar experiment with exponentially growing HL-60 cells where teniposide selectively killed S-phase cells (Gorczyca *et al.*, 1993).

Cytotoxicity of topoisomerase II inhibitors can be summarised as a complex phenomenon due to contribution of several different mechanisms (Fig.12). These mechanisms operate within varying time frames relative to exposure. Death caused by DNA damage and malfunction of essential genes is accompanied by reproductive death resulting from collision between persisting cleavable complexes and progressing replication forks (Qui *et al.*, 1996) or non-disjunction of sister chromatids during mitosis (Warburton and Earshaw, 1997). Considerable part of the cell population suffers extensive DNA damage and undergoes apoptosis (Roy *et al.*, 1992). Finally, a fraction of survived cells develops mutations leading to less viable phenotypes with eventual elimination of these cells from the population.

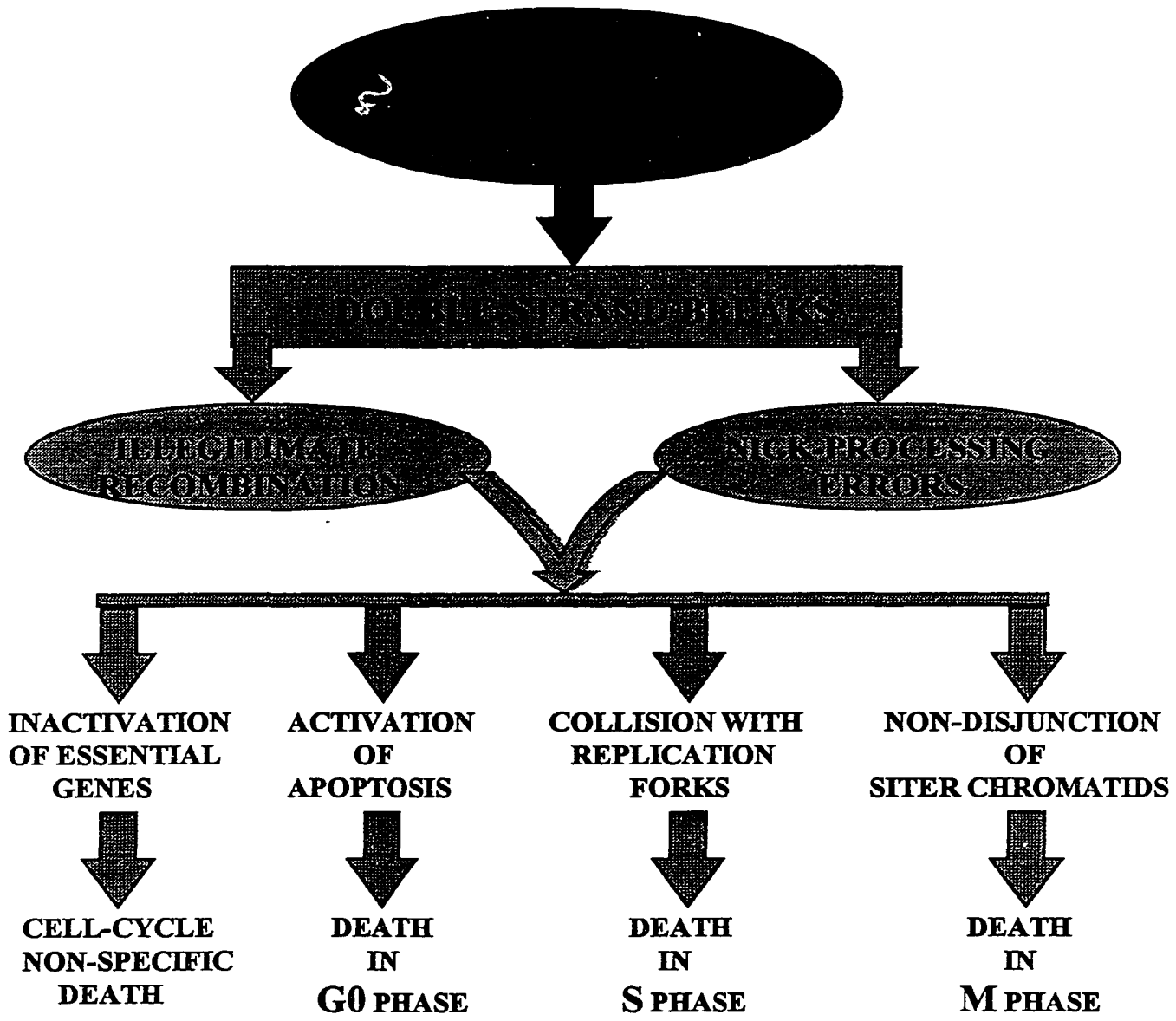

Figure 12. The mechanism of cytotoxicity of Topoisomerase II inhibitors

2.6 Mutagenicity

A potentially mutagenic consequence of exposure to DNA topoisomerase II inhibitors is induction of protein-linked double-strand breaks. Depending on a genetic target and/or cellular type, these breaks may cause mutation induction of different magnitude. In bacterial systems, non-intercalating agents fail to induce noticeable mutagenic response (Gupta *et al.*, 1987; Ashby *et al.*, 1994). On the other hand eucaryotes show a pronounced mutagenic response to non-intercalators (Gupta *et al.*, 1987; Singh and Gupta, 1983a, 1983b; Pommier *et al.*, 1985; Ashby *et al.*, 1994) the extent of which vary several orders of magnitude, depending on endpoints (Table 24).

Table 24 Mutagenicity of podophyllotoxins in mammalian cells

Cell type	Gene	Agent	Exposure dose[A]	hrs[B]	MF per 10^{-6}	Reference
CHO	*hprt*	VM-16 VM-26	1.25 0.12	16	1.3 *vs.* 45.6 2.8 *vs.* 115.3	Singh and Gupta, 1983
Mouse L	*hprt*	VM-16 VM-26	1 0.1	16	5 *vs.* 65 4 *vs.* 55	Gupta *et al.*, 1987
L5178Y *tk+/-*	*tk*	VM-16	0.5	1	0 *vs.* 6000	Evans *et al.*, 1989
CHO D422*	*hprt* *aprt*	VM-26	0.08 0.16	16	5 *vs.* 63 1.4 *vs.* 10.8	Han *et al.*, 1993
V79	*Cad ampl.*	VM-16	5 uM	1	5.8 *vs.* 1698	Di Leonardo *et al.*, 1993
L5178Y *tk+/-*	*tk*	VM-16	0.1	4	0 *vs.* 2300	Ashby *et al.*, 1994
V79*	*hprt*	VM-16	0.5	24	0.5 *vs.* 28	Hashimoto *et al.*, 1995
Hum. T-cells	*hprt*	VM-16	*	*	46 *vs.* 55	Karnaoukhova *et al.*, 1997
Hum. T-cells *TK6*	*Hprt* *hprt*	VM-16 VM-16	2 2	16 8	5.7 *vs.* 18.5 29 *vs.* 69	Khaidakov and Glickman, 1998, in preparation

(A) – maximal dose is in μg/ml; (B) - duration of exposure in hrs;
(*) – prior to experiment, cultures were treated for the elimination of pre-existing mutants or spontaneous MFs were subtracted;
MF in spontaneous cultures *vs.* drug-treated; in some cases values were deduced from graphs due to absence of numerical data.

Dimeric structure of enzyme, covalent bond with 5'-end of cleaved DNA, and abundance of topoisomerase II recognition sequences in the genome, create a situation favouring rearrangements in genome, resulting in deletions, insertions or duplications. At the same time, unlike ionizing radiation, inhibition of topoisomerase II does not result in DSBs with the modified bases at the site of DNA cleavage, which makes induction of point mutations less likely.

The most notable consequence of exposure to inhibitors of topoisomerase II is a high frequency of SCEs (Pommier et al, 1985). Topoisomerase II promoted recombination between two phage lambda DNA molecules with the formation of recombinant species carrying deletions or duplications (Bae *et al.*, 1988). In an experiment performed by Aratani *et al.*, (1996) *APRT* negative CHO cell transfected with plasmid carrying functional *APRT* gene were exposed to etoposide, teniposide and ICRF-193. After exposure, cell cultures were processed for selection of *APRT*-positive colonies. Compared to control cultures, exposed cells demonstrated 3-5-fold higher integration of plasmids into genome via non-homologous recombination. Similar results were reported for human cell lines PA1, HeLa and EJ-1 transfected with pSVneo plasmid in presence of etoposide, ICRF-193 and m-AMSA (Fujimaki *et al.*, 1996). In addition, inhibitors of topoisomerase II seem to stimulate illegitimate recombination mediated by other enzymes, as etoposide-treated human leukaemia cells exhibit significantly higher incidence of V(D)J-recombinase-mediated exons 2-3 deletions in *hprt* (Chen *et al.*, 1996).

The mutagenicity of topoisomerase II inhibitors in eucaryotes varies, depending on the selected end-point (Table 24). Considering the differences in the tolerance of

different assays to deletions, this is suggestive of involvement of large-scale rearrangements. For example, *hprt* has a relatively low tolerance for gross rearrangement allowing only recovery of deletions smaller than 3.5 Mb in size (Lippert *et al.*, 1995). *Aprt* gene in CHO D422+/0 cell line is even less tolerant, and fraction of deletions in both spontaneous and induced mutational spectra is apparently even smaller than in *hprt* (Meuth, 1992). The *tk* +/- system is more suitable in this regard, because *tk* +/- cells carry a double set of essential genes surrounding target-sequence. The mouse lymphoma assay (MLA) is able to tolerate deletions up to 1/4 of chromosome 11 in size (Ashby *et al.*, 1994) so that the potential for recovery of large deletions is much greater.

Exposure to epipodophillotoxins generally induced a relatively strong (1 order of magnitude) mutagenic response in the *hprt* gene *in vitro* (see Table 24). At the same time, surprisingly, the analysis of T-lymphocytes from cancer patients receiving chemotherapy treatment incorporating etoposide did not reveal any significant shifts in MF in post-treatment samples (Karnaoukhova *et al.*, 1997). Similarly, the lack of response in *hprt* was observed in CHO A_L cells subjected to amsacrine (Shibuya *et al.*, 1994), although in other *in vitro* experiments exposure to this inhibitor resulted in several-fold increase in *hprt* mutant frequency (Pommier *et al.*, 1985). The discrepancy in results is difficult to explain. In the case of *in vivo* study by Karnaoukhova *et al.*, (1997) this could reflect either differences in the response between dividing and non-dividing cells, or extended exposure (weeks *vs.* hours) resulting in the killing of the majority of sensitive cells.

In contrast to hemizygous systems, the selection for *TK*-mutants after exposure to inhibitors in *tk* +/- L5178Y cells resulted in a 1000-fold mutant induction (Evans *et al.*,

1989; Ashby *et al.*, 1994). Such an increase in mutant frequencies has been provided almost exclusively by slow-growing colonies (Ashby *et al.*, 1994). The slow growing phenotype is thought to be a result of large rearrangements, removing from one of the chromosomes a set of essential genes, the event that presumably hampers cellular growth.

2.7 Mutational spectrum

The mutational specificity of podophillotoxins if far from clear, even though their pronounced ability to induce gross rearrangements seems to be well established. The analysis of etoposide-induced large and small mutant colonies in the mouse lymphoma L5178Y cells revealed that 83% and 92% respectively had lost the 6.3 *tk*+ fragment (Ashby *et al.*, 1994). It should be noted however, that loss of the 6.3 fragment may be observed in up to 82% of spontaneously arising mutants as well (el-Tarras *et al.*, 1995). This may indicate on genetic instability in L5178Y cells characteristic for tumour cells in general (Orth *et al.*, 1994; Lengauer *et al.*, 1997). Therefore the observed increase in mutant frequency could be a reflection of **non-specific** enhancement of genetic instability caused by the presence of cytotoxic agent.

In *aprt* hemizygous (+/0) CHO cells, the podophillotoxin-induced mutational spectrum was substantially different from that seen in the mouse lymphoma assay. The exposure of the D422 line to teniposide (VM-26) resulted in the increase in small deletions (29% *vs.* 21%) as well as insertions (23% *vs.* 7%) and large deletions (11% *vs.* 0%) totalling 63% of the entire mutational spectrum (Han *et al.*, 1993). Interestingly, strong topoisomerase cleavage sites tended to cluster within small deletions, although the relative position of these sites towards deletion breakpoints varied. Ripley (1994) offered

a unified nick-processing model allowing a feasible connection between topoisomerase II cleavage and the possible mechanism of formation of the majority of the recovered deletions and duplications. It is hypothesised that they are the result of either the sole or joint activity of polymerase and/or 5'-nuclease.

In terms of tolerance to deletions, the *hprt* gene is between the heterozygous *tk* and homozygous *aprt*. The molecular analysis of a small number of spontaneous and etoposide-induced *hprt* mutants revealed that all three spontaneous mutants had normal restriction pattern, whereas 9 of 10 induced mutants had partial (3) or total (6) deletions of the gene (Berger *et al.*, 1991). In contrast, in a large *in vivo* study on T-lymphocytes in cancer patients on etoposide chemotherapy, there was no increase either in deletions or insertions (Karnaoukhova *et al.*, 1997). The only significant change in the mutational spectrum was a small increase in frequency of A:T to T:A transversions at the expense of G:C to T:A events. It should be noted, however, that gross rearrangements were observed in most studies on podophillotoxins (Ashby *et al.*, 1994; Han *et al.*, 1993; Berger *et al.*, 1991), as well as other inhibitors interfering with cleavage-religation reaction of topoisomerase II (Pommier *et al.*, 1985; Shibuya *et al.*, 1994). The formation of large-scale deletions is attributed to a sub-unit exchange between different enzyme-DNA complexes triggering illegitimate recombination (Pommier *et al.*, 1985), a mechanism initially proposed for bacterial gyrase-mediated events (Ikeda *et al.*, 1982).
Another consequence of topoisomerase II inhibition is the large-scale amplification of genomic sequences. It has been shown that topoisomerase II deficient cells exhibit amplification-prone phenotype (Di Leonardo *et al.*, 1992). In experiments with V79 cells subjected to nalidixic acid (NA), NA-resistant cell lines showed lower concentration of

enzyme as well as cross resistance to other enzyme inhibitors including etoposide. In all these lines, the frequency of clones resistant to inhibitors of CAD, DHFR, and IMPDH genes products (a phenotype caused by overproduction of gene products due to gene amplification) was considerably enhanced. Similarly, treatment of parental V79 cells with etoposide produced about 100-fold increase of PALA (inhibitor of aspartate transcarbamylase activity of the CAD gene) resistant mutants with the CAD gene amplification confirmed by dot-blot hybridization (Di Leonardo *et al.*, 1993). It is hypothesised that chromosomal aberrations, typically caused by topoisomerase II inhibitors, may initiate gene amplification by introducing DNA strand breaks into the "replication bubble", and the generation of acentric elements (Windle *et al.*, 1991; 1992).

3. Analysis of Mutagenesis of Etoposide in Human T-lymphocytes and TK6 cells.

3.1. ABSTRACT

The *in vitro* mutagenic response of primary human T-lymphocytes cells to etoposide at the *hprt* locus was analysed. Freshly isolated T-cells (G_0 phase) or T-cells grown for 7 days (log phase) were exposed to etoposide at clinically relevant doses (1 and 2 μg/ml for 16 hrs). Exponentially growing non-exposed and exposed cultures were processed using the *HPRT* clonal assay, and their plating efficiencies (PE) and mutant frequencies (MF) determined. *HPRT*⁻ mutants were isolated for further DNA sequence analysis. Background MFs for G_0 and Log phase T-cells were 5.7 +/- 2.7 and 11.0 +/- 2.6 per 10^6 cells, respectively. Upon exposure of G_0 T-lymphocytes an increase in MF at both concentrations was observed (1 μg/ml - 16.6 +/- 4.1, p=0.024; 2 μg/ml - 18.5 +/- 9.8). In contrast, log-phase cells did not exhibit any increase in MFs in response to etoposide exposure (1 μg/ml - 8.1 +/- 2.4; 2 μg/ml - 8.6 +/- 1.0). Mutational spectra in spontaneous and etoposide-treated Log cultures were very similar, whereas G_0 phase cells exhibited 2-fold increase in splice errors at the expense of base substitutions.

TK6 cells were treated with etoposide using an 8-hr exposure protocol at D_{37} and D_{10} doses. TK6 cells did not react at D_{37} dose of etoposide and showed marginally significant mutagenic response (p=0.04) at the D_{10} dose with slightly over 2-fold increase in *hprt* mutant frequency. Mutational spectra in all groups were heavily dominated by splice errors and were not analysed for the differences. However, fraction of deletions detectable by multiplex PCR in D_{10} group has been ~50% higher than in control. Obtained data suggest that there is a difference between quiescent and proliferating T-lymphocytes, as well as between proliferating T-lymphocytes and TK6 cells in their reaction to etoposide exposure with respect to both mutagenic response and possibly dominating mechanisms of mutagenesis.

3.2 Introduction

In recent years, several novel classes of antitumor agents have been developed. Among them, agents interfering with the action of topoisomerases have gained considerable popularity due to their high effectiveness. Topoisomerases alter topological structure of DNA and relieve torsional stress by creating DNA strand breaks. They form reversible cleavable complexes with DNA covalently linked to 5'-prime ends of breaks, and remove supercoils by passage of strands and consequent resealing of breaks. There are two main types of enzyme in mammalian cells (Wang, 1985). Type I topoisomerases introduce only single-strand breaks that permit the passive relaxation of DNA. Type II enzyme creates double-strand breaks allowing decatenation, and segregation of newly synthesised chromosome).

Several inhibitors of topoisomerase II including etoposide act through the stabilisation of cleavable complexes and the inhibition of strand passage/ ligation activity

of enzyme (Ferguson and Baguley, 1994; Anderson and Berger, 1994). As a result, exposure to these agents leads to substantial increase in double-strand breaks, SCEs, chromosomal aberrations, mutation induction and cell-killing (Singh and Gupta, 1983a; Pommier *et al.*, 1985,1988). These effects are correlated with the cell-cycle specific changes in cellular topoisomerase II concentration (Chow and Ross, 1987; Sullivan *et al.*, 1987a; Sullivan *et al.*, 1987b; Webb *et al.*, 1991), which is highest in S and G_2 phases of the cell cycle.

High cytotoxicity and genotoxicity of topoisomerase II inhibitors in proliferating cells are undoubtedly the main determinants of their chemotherapeutic efficiency. At the same time, the mutagenicity of these agents increases the probability of treatment-induced secondary malignancies. Indeed, it has been reported that chemotherapy regimes including the epipodophylotoxin etoposide result in high incidence of acute myelogenous leukemia, and other forms of cancer (Pedersen-Bjergaard and Philip, 1991; Ratain *et al.*, 1987). The subject of the present study was to analyze mutagenicity and mutational spectrum induced by a derivative of epipodophylotoxin etoposide in primary T-lymphocytes.

3.3 Materials And Methods

Etoposide (4'-demethylepipodophylotoxin ethylidine glucoside, VP-16) was a generous gift from Bristol Meyers Squibb. Fresh blood samples were taken from male volunteers (staff members of CEH, UVIC), and the mononuclear cells were isolated using leukoprep tubes (Hycore). Isolated mononucleocytes were washed twice in RPMI-1640 supplemented with 10% CBS, and cultured in medium for 5-7 days. Growth medium

consisted of RPMI 1640 (Hyclone), 20% HL-1(Ventrix), 5% calf serum (Professional Diagnostics), 5% human AB serum (Gibco/BRL), 5U/ml IL-2 (Cellular Products Inc.), 0.25 mg/ml PHA (Wellcome), 2 mM L-glutamine, 2 mM pyruvic acid, 100 units/ml penicillin, 100 mg/ml streptomycin (Sigma) and 4% Fungizone (Gibco). Proliferating lymphocyte cultures were then divided into aliquots (2-5 mln cells) and treated with clinically relevant, 1 or 2 ug/ml, concentrations of etoposide for 16 hrs (Splinter *et al.*, 1992; van der Gaast *et al.*, 1992). After a 7-days expression period required prior to *hprt* mutant selection, exposed and non-exposed T-cells were plated in selective and non-selective media in microtitre plates (96 well, flat bottom). Cells were plated at a density 10^4 and 3 cells per well for selective and non-selective plates respectively, along with 10^4 lethally irradiated feeder cells (RJK 853 lymphoblastoid cells derived from a Lesch-Nyhan patient having a complete deletion of the *hprt* gene). Selection plates contained 10^{-5} M 6-thioguanine (Sigma). Plates were incubated on a sloped shelf (5°) in a 5% CO_2, 37°C, humidified incubator for a period of 14 days. Twenty-four hours prior to scoring, the plates were rotated 180° on the sloped shelf then scored visually under an inverted phase contrast microscope for wells that contained expanding colonies. *HPRT* mutant frequencies were determined as a ratio between cloning efficiencies in selective and non-selective conditions. Mutant colonies were transferred to a 24 well flat bottom dish with 2 ml of fresh selective media and expanded for the analysis of genomic DNA.

Lymphoblastoid TK6 cells were routinely maintained in culture in growth medium essentially identical to that for T-lymphocytes with the exception of IL-2 and PHA. The cytotoxicity of etoposide in TK6 cells was determined using 8 hrs exposure protocol at cell density of 2×10^5 cells/ml. TK6 cells were exposed to etoposide at 0, 0.125, 0.25, 0.5, 1.0,

2.0, and 5.0 μg/ml, and plated in 96 microtitre plates at a density 3 cell/well immediately after the removal of drug from the medium. Survival was determined after 14 days of incubation as a ratio of positive colonies between control and etoposide-treated cultures. A mutagenicity study of etoposide in TK6 cells was carried out using the same protocol and D_{37} and D_{10} doses of etoposide (0.125 and 1.0 μg/ml, see figure 14). Control and exposed cultures were grown for 7 days for phenotypic expression and then put through the *Hprt* assay (see above) without feeder cells. In four independently maintained and treated cultures 10 mutants were randomly selected from each group and used for further analysis. Amplification of the 8 fragments from genomic *hprt* sequence (multiplex PCR analysis) including all 9 exons plus some flanking intronic sequences was performed essentially as described by Gibbs *et al.* (1990). Crude cell extracts preparation from about 10^4 cells per reaction was carried out as described by Fuscoe *et al.* (1992a). Amplification of *HPRT* cDNA from cellular mRNA (RT-PCR assay) of mutant clones was carried out in two consecutive PCR reactions using nested primers (Yang *et al.*, 1989), and the recovered cDNAs were sequenced on a Pharmacia A.L.F. or a Li-Core automated sequencer according to the manufacturer's instructions.

3.4 Results

Exposure of T-lymphocytes to etoposide at clinically relevant doses did not have any mutagenic effect on the Log-phase cultures (Fig.12). Spontaneous mutant frequencies ranged from 4 to 19.3 x 10^{-6} mutants with the mean value of 11.0 ± 2.6, whereas MF values in groups treated with 1 μg/ml and 2 μg/ml etoposide were 8.1 ± 2.4 and 8.6 ± 1.0 x 10^{-6} respectively. Spontaneous mutant frequency in freshly isolated lymphocytes was 2-fold lower and constituted 5.7 ± 2.7 mutants per million cells. Exposure of G_0 cells to etoposide resulted in a 3-fold increase of mutant frequency, which was highly significant at the lower dose of drug of 1 μg/ml - 16.6 ± 4.1, p=0.024.

Mutants from three samples in each group were analysed at the molecular level (Table 25 and 26). Spontaneous mutational spectrum consisted of 55.6% base substitutions, 32% splice errors, 4% frameshifts, 8% deletions, and 4% insertions (Table 25). This is very similar to the T-lymphocyte spontaneous mutational spectra derived from *HPRT* database (Cariello *et al.*, 1994), and the spontaneous mutational data generated in our laboratory (Curry *et al.*, 1995). One of deletions (ZoSP10) spanned exons 2-8 and was flanked by 4 bp direct repeats, whereas the other deletion (GS_0SP4) did not have any repeats and was located within exon 8.

Distribution of mutations by class in etoposide-treated log-phase cultures was rather close to that in spontaneous data-set and consisted of 44% base substitutions and 36% splice errors. Deletions were not detected, although there was some increase in complex mutations. On the contrary, mutants isolated from G_0 cells exposed to etoposide exhibited 2-fold higher percentage of splicing errors, some increase in incidence of complex mutations, and almost 3-fold decline in the level of base substitutions, with no

significant changes in incidence of frameshifts or deletions. Multiplex PCR analysis has not revealed any differences in percentage of mutants with visible rearrangements in all groups (not shown).

TK6 cells were treated with etoposide for 8 hrs at doses producing approximately 60% and 90% cell killing (0.125 and 1 μg/ml respectively, Fig.13). Upon exposure TK6 cells exhibited a slight (less than 2-fold) increase in mutant frequencies at highest dose (Fig.13, p=0.04), accompanied with about 50% increase in gross rearrangements detectable by mPCR (Tables 28 and 29). Vast majority of mutants in all groups had exclusions of one or several exons. Also, there was a slight excess of deletions in the dataset from cultures exposed to etoposide at D_{10} dose., although it should be noted that some of these deletions were also found in spontaneous groups.

Several mutants in all groups yielded cDNA with large deletions with the breakpoints clustered in exons 2 and 8, and surrounded by direct repeats (see legend to Table 28). In most cases, reaction mixtures contained two PCR products, with the second product carrying various mutations, mostly splice errors, always spanning smaller region. Also, these deletions were not paralleled by changes in mPCR patterns, which showed presence of all nine exons in genomic DNA.

3.5 Discussion

Generally, hemizygous *hprt* shows far poorer (10-100 times) mutagenic response to clastogens than more tolerant to large-scale deletions/rearrangements somatic genes (Evans *et al.*, 1986; Amundson and Liber, 1991). Yet a certain increase in mutant frequencies as well as specific changes in mutational spectra can be detected, although

results from different laboratories are sometimes contradictive and mutually exclusive. For example, exposure of Chinese Hamster V79 cells to etoposide at 68-28% survival concentrations resulted in several-fold (>30-fold at D_{28}) increase in *HPRT* mutant frequencies (Singh and Gupta, 1983a, 1983b). Similar results were obtained by Pommier *et al.*, (1985) with V79 cell treated with amsacrine, and Han *et al.*, (1993) with CHO-422 aprt +/0 cells exposed to etoposide. In several other studies using amsacrine in various *in vitro* mammalian mutation systems (Pommier *et al.*, 1985; Moore *et al.*, 1989) *hprt* also showed a certain mutagenic response although much smaller than in *tk* gene. It should be noted, however, that being an intercalator, this inhibitor is capable of producing frameshifts, which probably account for a sizeable part of mutant induction. On the other hand, there was no clear mutant induction observed in *hprt* of CHO A_L cell line exposed to amsacrine (Shibuya *et al.*, 1994), as well as in the *in vivo* study of cancer patients receiving several courses of etoposide treatment (Karnaoukhova *et al.*, 1997).

Our results indicate that etoposide is only weakly mutagenic in G_0 T-lymphocytes, and non-mutagenic in exponentially growing T-cells (Fig.12). In contrast to dividing T-cells, Log phase TK6 cells showed modest mutant induction at the most cytotoxic dose ($p=0.04$, Fig.13).

The difference in reaction between G_0 and Log phase T-cell cultures can be attributed to the content of topoisomerase II and consequent shifts in balance between DNA damage, cytotoxicity and mutagenesis in quiescent and proliferating cells. Etoposide-induced DNA damage correlates with cell-cycle specific fluctuations of topoisomerase II activity (Sullivan *et al.*, 1987ab) which is highest in S and G_2 phases of the cell cycle. Similarly, the cytotoxicity of etoposide is also cell cycle specific. In

several experiments it has been shown that etoposide is highly cytotoxic and genotoxic for dividing cells and virtually harmless for non-dividing or quiescent cells (Sullivan *et al.*, 1986; Zwelling *et al.*, 1987; Markovits *et al.*, 1987).

The distribution and content of topoisomerase II in the chromosomes vary significantly in the different stages of the cell cycle (Warburton and Earshaw, 1997). For example, topoisomerase II is evenly distributed throughout prophase chromosomes, restricted to centromeric region at metaphase, and disappears during anaphase when segregation is complete (Sumner, 1996). In the interphase nucleus, topoisomerase II is also uniformly distributed throughout the chromosome, and apparently localised at the sites where it is required (Swedlow *et al.*, 1993). In Go human lymphocytes, the enzyme is about 100-fold lower than in PHA-stimulated cells (Hwang *et al.*, 1989). It is likely that in the resting cells topoisomerase molecules are spaced either very widely, or in a very close proximity (between or within constitutively expressed genes). It can be speculated that subunit exchanges in Go cells would lead to either very large-scale rearrangements (incompatible with life) or the small intragenic deletions/insertions.

Inhibitors of topoisomerase II are also known to trigger apoptosis in various types of cells including thymocytes (Walker *et al.*, 1991), CHO cells (Lock and Ross, 1990; Chatterjee *et al.*, 1990), lymphoblastoid cells (Morris *et al.*, 1995), and T- and B-lymphocytes (Fournel *et al.*, 1995). An additional mechanism of cytotoxicity present in proliferating cells and absent in resting cells is a collision of replication forks with the drug-stabilised cleavable complexes (Catapano *et al.*, 1997). Therefore, cytotoxicity of etoposide in G_0 cells is several-fold lower allowing a larger fraction of affected cells to participate in mutagenesis. But, perhaps, the main factor determining mutagenic outcome

in these groups is a state of the cell populations. Vast majority of freshly isolated lymphocytes is in G_0 phase, whereas in exponentially growing cultures cells are at different stages of the cell cycle. There is a very substantial difference between resting and dividing cells with respect to recombination activity (Thyagarajan *et al.*, 1996), activity of repair genes (Donohue *et al.*, 1996; Yamamoto *et al.*, 1996), or volume of deoxyribonucleotide pool available for repair (Green *et al.*, 1996). Therefore, it is possible that mutagenicity and even mutational specificity of etoposide may be quite dissimilar in quiescent and dividing cells.

The discrepancy in mutagenic response between proliferating T-cells and lymphoblastoid TK6 cells may be also related to the differences between various cell types in their reaction to a massive DNA damage. Immortalised or tumor cell lines exhibit an array of unusual properties including disregulation of apoptosis. This may happen via inactivation of genes responsible for cell arrest (p53, Rb, White *et al.*, 1994), or viral incorporation of genes interfering with execution of apoptotic program (Wyllie, 1995). Compared to cells with finite lifespan these cells may demonstrate significantly higher survival. Also, compared to normal cells, transformed cells typically maintain higher concentration of topoisomerase II, which becomes less sensitive to the internal regulatory signals (Hsiang *et al.*, 1988). Therefore, it is possible that doses of etoposide applied to T-lymphocytes were not equal in toxicity to a D_{10} dose, which (unlike D_{37} dose) did produce a certain mutagenic response in TK6 cells.

In our etoposide-induced mutational spectra we did not observe drastic shifts in incidence of gross rearrangements or small deletions or insertions (Tables 25-29, see results section). However, there was a certain increase in both large-scale events

detectable by the multiplex PCR method and small deletions in TK6 cells. In the mutant dataset from cultures exposed to D_{10} dose of etoposide percentage of abnormal mPCR patterns comprised 33% was about 50% higher than in control group (22%). Also, there were three novel cDNA deletions ranging from 3 to 24 nucleotides in size. Although breakpoints of these deletions were located in the close proximity to potential topoisomerase II cleavage sites (Fig. 4) other possible mechanisms of their formation could not be ruled out. For example, a 3 bp deletion ATT in the mutant TK91257 is flanked by two base-pair AT repeat suggesting slippage-misalignment. Similarly, deleted sequence TTAA (mutant TK812) is capable of forming a hairpin structure, which could be responsible for this event. Interestingly, treated G_0 phase T-lymphocytes exhibited a considerable (2-fold) increase in splice errors (Table 26), which could be a reflection of topoisomerase II poisoning. Introns are typically AT-rich and contain numerous potential topoisomerase II consensus sequences (Fig. 15). It is quite likely that formation of deletions and insertions in legitimate splice consensus sequences due to faulty nick-processing would result in higher yield of splice errors. The fact that some of topoisomerase II cleavage sites are positioned directly within splice sites of exons 1, 3, 4, 6, 7, and speaks in favour of this assumption. In TK6 cells splicing errors heavily dominated mutational spectrum in all groups, which probably masked shifts caused by exposure to etoposide.

As follows from data in literature, mutational specificity of inhibitors of topoisomerase II appears to be dependent on properties of selected genetic target, TOPO II inhibitor and probably experimental protocol. In a small *in vitro* study on Chinese Hamster V79 cells (Berger *et al.*, 1991), 9 out of 10 isolated *HPRT* mutants from

etoposide-treated cultures showed partial deletions or rearrangements in *hprt* while all 3 spontaneous mutants had Southern blots consistent with the wild-type restriction pattern. Treatment of hybrid (CHO + human) A_L cell line (which is virtually limitlessly tolerant to deletions) with another inhibitor of topoisomerase II - amsacrine also induced mostly large deletions (92%) of at least 1.5 -2 Mb in length (Shibuya *et al.*, 1994). This type of rearrangements in genome is attributed to a sub-units exchange between two dimeric topoisomerase II-DNA complexes. Exposure of aprt +/0 CHO D422 cells to teniposide resulted mostly in accumulation of small (1-20 bp) deletions and insertions clustered in regions with sites for topoisomerase II- mediated DNA cleavage (Han *et al.*, 1993). Their formation is thought to be a result of incorrect nick processing (Ripley, 1994) favouring inclusion or exclusion of several nucleotides at the position of cleavage.

One of the peculiar findings in this study was an occurrence of large cDNA deletions not accompanied by corresponding changes in genomic DNA. These deletions typically involved exons 2-8 and were flanked by direct repeats of various lengths (see legend for Table 28). The majority (but not all) of mutants concerned yielded a mixture of PCR products, with the second product carrying different types of mutations. All the above indicates that the most likely source of these apparently artificial events is RT-PCR reaction, namely its first step. Analysis of secondary structure of *hprt* mRNA at 37^0C (Fig.14) revealed that at least in two cases, segments containing these repeats are brought into close proximity. It can be speculated that under these conditions, misalignment was greatly facilitated and reverse transcriptase could synthesise a short cDNA product carrying large deletion. In the course of further amplification, this product could become dominant in the reaction mixture due to its higher turnover rate.

Based on the obtained results we can conclude that mutagenic responsiveness of T-lymphocytes to etoposide is likely dependent on resting or dividing state of the cells in the population. Discrepancy in mutagenicity of etoposide between exponentially growing T-cells and TK-6 cells could be either a result of unequal cytotoxicity of drug at applied doses or caused by different susceptibility to apoptosis.

Indeed, the role of apoptosis as modulator of cellular mutagenic response to challenges exceeding a certain level is becoming increasingly clear. There are several reports showing that cell lines with differing susceptibility to apoptosis have dramatically dissimilar mutant induction after exposure to mutagens (Phillips *et al.*, 1995; Morris *et al.*, 1996). In the more controlled experimental conditions, cells with stably overexpressed Bcl-2 protein subjected to ionizing radiation also showed 1.5-2-fold higher *hprt* mutant frequency compared to the cells transfected with the vector without Bcl-2 (Cherbonell-Lassere *et al.*, 1996). Similarly, WTK1 human lymphoblastoid cells containing mutant p53, exhibit lower radiosensitivity as well as higher mutagenic response, compared to closely related TK6 cells with wild type p53 (Phillips *et al.*, 1995). At the same time, there was no difference in mutational spectra between two lines (Phillips *et al.*, 1995) suggesting that apoptosis is an essentially indiscriminate process eliminating cells with either a lower threshold for apoptosis induction or relatively higher DNA damage.

To test the possible contribution of apoptosis to our results observed *in vitro* with etoposide, we conducted experiments on TK6 cells using cycloheximide as a suppressor of apoptosis. Our choice of inhibitor was based on previous studies indicating that cycloheximide indeed prevents apoptotic death in different types of cells exposed to

etoposide (Chow *et al.*, 1988). In our experiment, presence of cycloheximide in the medium also significantly improved cloning efficiency ($p = 0.02$, Fig.18). However, the improvement in survival was not matched by an increase in mutant frequency (Fig. 18 and 19) as was expected under the above assumption. In fact, in all groups treated with cycloheximide, MFs were lower than in corresponding groups without treatment. This suggests that in the case of etoposide, apoptosis does not modulate mutagenic response, although there also may be several additional considerations regarding cycloheximide as a powerful inhibitor of both DNA and RNA synthesis (Gokal *et al.*, 1986), and its possible effects on repair and recombinational activity (Sono and Sakaguchi, 1984). It should be noted, however, that our findings are supported by results obtained in a similar experiment with V79 Chinese hamster cells transfected with expression vector carrying Bcl2 cDNA (Hashimoto *et al.*, 1995). Upon treatment with etoposide, cells overexpressing Bcl2 demonstrated increased survival accompanied with lower induction of SCEs and *hprt* mutants. This suggests that regardless of method employed, apoptotic suppression diminishes mutagenicity of etoposide probably via intervention between formation of cleavable complexes and machinery involved in recombination.

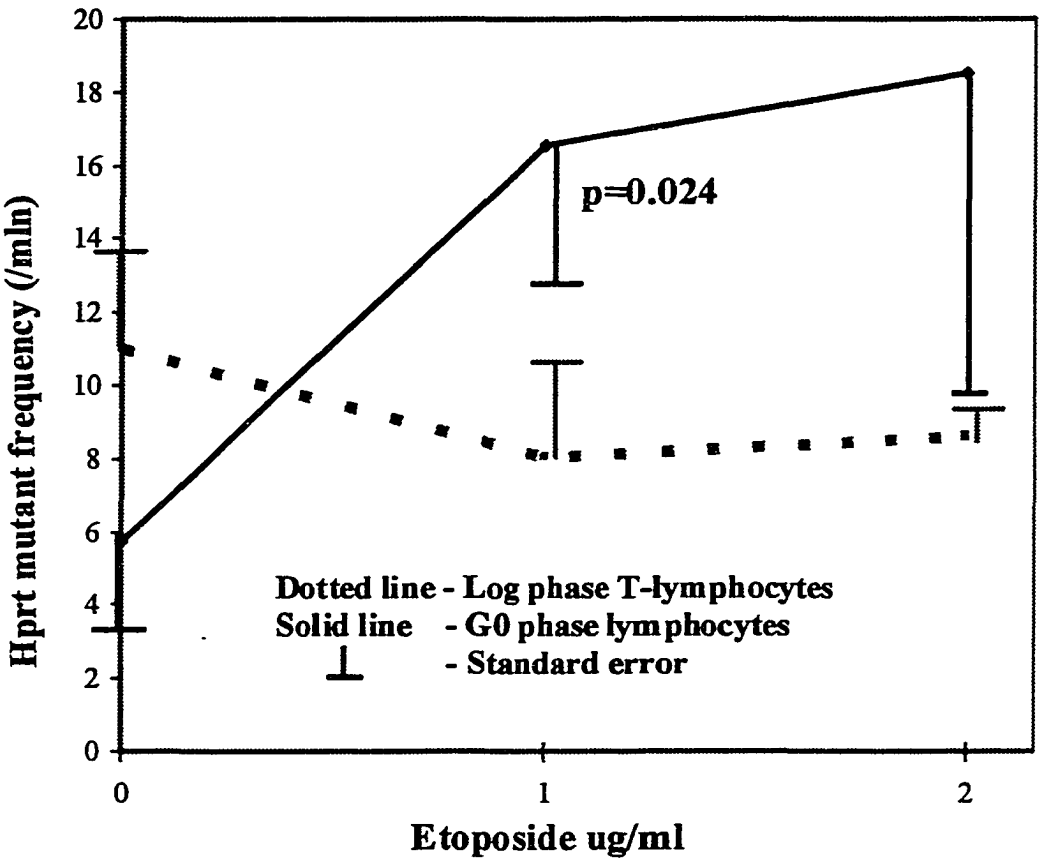

Figure 13. Mutagenicity of etoposide in Log and G0 phase T-lymphocytes
Legend: Go and Log phase T-lymphocytes were exposed to etoposide at therapeutic concentrations for 16 hours.

Table 25. Spontaneous *HPRT* cDNA mutations recovered in T-lymphocytes.

MUTANT	MUTATION	POSITION	SEQUENCE	mPCR
B_0SP-3	T→ C	125	GACTA ..c.. TATGG	WT
B_0SP-6	Exons 2-6 loss			WT
GS_0-SP-4	Deletion	568-573	GTTGTC <ggatat>GCCCTT	WT
Uo-SP-1	Exons 2-3 loss			NA
Uo-SP-15	G → A	617	TGTTT ..a.. TGTCA	WT
Z_0-SP-1	T → G	548	TGAAA ..g.. TCCAG	WT
Z_0-SP-3,11	Exons 2-3 loss			2,3
Z_0-SP-8,13	Exons 2-3 loss			3
Z_0-SP-4	Exon 4 loss			WT
Z_0-SP-7,9	G → A	209	CAAGG ..a.. GGGCT	WT
Z_0-SP-10	Deletion	93-570	GGAGA <tttgg.. .. gga> TATGCCCTTG	NA
Gsp-1,4,20,24,31	C → G	610	TATAG ..c.. ATGTT	WT
Gsp-5,11,26	Exon 8 loss			WT
Gsp-6,15,19,23,27	Missing A	340	GGGAC .. .. TAAAA	WT
Gsp-9	C → A	222	AAATT ..c.. TTTGC	WT
Gsp-18	G → A	617	TGTTT ..a.. TGTCA	WT
Gsp-28	G → C			WT
Gsp-35	G → A	272	TGATA ..a.. ATCCA	WT
Msp-5	G → T	625	CATTA ..t.. TGAAA	WT
Msp-9	C → T	225	CAATG ..t.. AGACT	WT
Msp-13	Exon 3 loss			WT
Q_0SP-6	A → C	602	CAGGG ..c.. TTTGA	WT
Q_0SP-7	Exons 2-3 loss			WT
Q_0SP-13,15	T → G	104	AAGGG ..g.. GTTTA	WT
Wsp-2	C → A	594	GAATA ..a.. TTCAG	WT
Wsp-3	Exons 2-3 loss			WT
Wsp-6	Insertion		Ex.8 <AATATAATAG> Ex.9	WT
Wsp-7,8,9,10,15	A → T	581	CCTTG ..t.. CTATA	WT

Legend: Mutants isolated from G_0 and exponentially growing cultures are combined. Mutants from the same culture with identical mutation are listed as one, unless multiplex PCR pattern showed otherwise.

Table 26. *Hprt* cDNA mutations recovered in G_0 and Log phase T-lymphocytes treated with etoposide

MUTANT	MUTATION	POSITION	SEQUENCE
Go phase			
GS_0-E1-2,3	T → G	613	GCATG..g..TTGTT
GS_0-E1-8	A → T	59	CCTTG ..t.. TTTAT
GS_0-E1-9	Exons 2-3 loss		
GS_0-E1-12,13	Deletion	44-131	GAAC <cagg.. ..tgga> CAGGAC
GS_0-E2-1,5,7,9	Exon 8 loss		
GS_0-E2-6	Exons 4-5 loss		
GS_0-E2-8	G → A	617	TGTTT ..a.. TGTCA
X_0-E1-1	Exons 2-6 loss		
X_0-E1-2	Exons 2-6 loss and T → C	615	ATGTT ..c.. GTGTC
X_0-E1-7	Exons 2-4 loss		
X_0-E1-9,15,19,22	Exon 8 loss		
X_0-E1-13	Exons 2-6 loss		
X_0-E1-14,20,21	Exons 2-3 loss		
X_0-E1-18	Extra T	614-616	ATGTT ..t.. TGTGT
Z_0-E1-2	Exons 2-6 loss		
Z_0-E1-5	G → A	255	GCACT ..a.. AATAG
Z_0-E1-6	Exons 2-4 loss		
Z_0-E1-8,9	Exons 2-3 loss		
Z_0-E1-11	Exons 2-6 loss and G → T	500	GAAAA ..t.. GACCC
Z_0-E2-2	Exon 5 loss		
Log phase			
GE1-6	T → C	533	AGACT ..c.. TGTTG
GE2-3,11	G → A	134	GGACA ..a.. GACTG
GE1-10	Exons 2-3 loss		
GE1-12	Insertion 66bp	403	aaagG..GCAAG...AAAGG...ATATA
GE2-18	Exon 3 loss		
GE2-19	C → T	151	TTGCT ..t.. GAGAT
ME1-4,6,9	Exon 6 loss		
ME1-5	Exons 1-4 and 6 loss		
ME2-1,6,8	Missing A	496	GTGAA .. .. AGGAC
ME2-3,5	Intact cDNA		
ME2-10	C → A	222	AAATT ..a.. TTTGC
PKSE2-4	T → G	146	ACGTC..g.. TGCTC
PKSE2-8	Exons 2-3 loss		
WE1-1	A → T	590	TAARG ..t.. ATACT
WE1-2	G → A	47	ACCAG ..a.. TTATG
WE1-3,10,16,20,22	Exon 4 loss		
WE1-4	G → T	580	CCCTT ..t.. ACTAT
WE1-7	Exons 4-8 loss		
WE1-11,18,25	A → T	581	CCTTG ..t.. CTATA
WE1-13	G → A	606	GATTT ..a.. AAT
WE2-2	Exons 2-6 loss		
WE2-5,10	Exons 2-3 loss		
WE2-14	Exons 2-8 loss		
WE2-16	Exons 2-6 loss and G → T	626	CATTA ..t.. TGAAA
WE2-19	G → T	626	CATTA ..t.. TGAAA

Table 27. *Hprt* cDNA mutational spectra in control and etoposide-treated T-lymphocytes.

MUTATIONS BY CLASS		SPONTANEOUS		INDUCED G_0 PHASE		INDUCED LOG PHASE	
		N	%	N	%	N	%
TRANSITIONS	GC→AT	5	17.8	2	10	4	16
	AT→GC	1	3.6	0	0	1	4
TRANSVERSIONS	GC→TA	3	10.7	0	0	3	12
	GC→CG	2	7.2	0	0	0	0
	AT→TA	1	3.6	1	5	2	8
	AT→CG	3	10.7	1	5	1	4
TOTAL BASE SUBSTITUTIONS		15	53.6	4	20	11	44
SPLICE ERRORS		9	32.1	12	60	9	36
FRAMESHIFTS		1	3.6	1	5	1	4
DELETIONS		2	7.2	1	5	0	0
INSERTIONS		1	3.6	0	0	1	4
COMPLEX		0	0	2	10	2	8
NO MUTATION		0	0	0	0	1	4
TOTAL		28/46	100	20/31	100	25/38	100

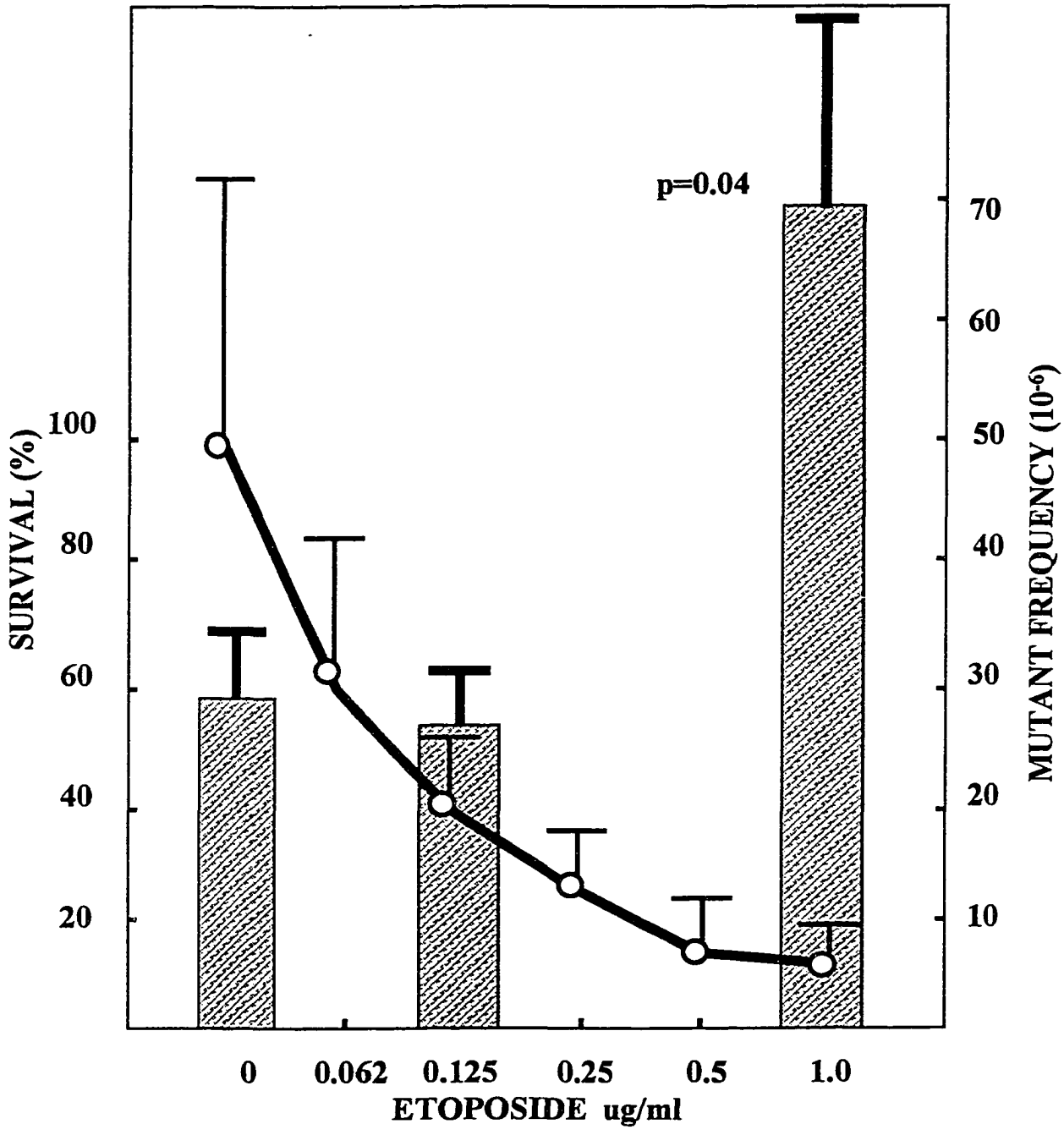

Figure 14. Cytotoxicity and mutagenicity of etoposide in TK6 cells
Legend: Cytotoxicity and mutagenicity have been determined in four separate experiments by exposing TK6 cells to D_{37} and D_{10} doses for 8 hours.

Table 28. *Hprt cDNA* mutations recovered in spontaneous TK6 cultures.

MUTANT	MUTATION	POSITION	SEQUENCE CONTEXT	mPCR
TK7-0-1	Ex. 2 p.del	31-34	Ex.1 [ATTAG] TGATG	WT
TK7-0-2	Ex. 2-6 loss			WT
TK7-0-3	Ex. 2-6 loss			WT
TK7-0-4	Deletion	33-146	ATTAG [TGATG.. ..CGTCT] TGCTC	Δ E9p
TK7-0-5	Ex.2-5 loss			WT
TK7-0-6	Ex. 2-6 loss			WT
TK7-0-7[A]	Ex.5 loss			WT
TK7-0-8	-			WT
TK7-0-9	Ex.4-8 loss			WT
TK7-0-10	Ex. 2-6 loss			WT
TK8-0-1	Ex.2-3,6 loss			WT
TK802	-			Δ E3
TK8-0-3	Ex. 2-6 loss			WT
TK8-0-4	Ex.2-3 loss			WT
TK8-0-5	-			Δ E7-9
TK8-0-6	Ex. 2-6 loss			WT
TK8-0-7	Ex.2-3 loss			Δ E3
TK8-0-8	Ex.2-8 loss			Δ E6-8
TK8-0-9	Ex. 3-8 loss			Δ E7-8
TK8-0-10	Ex. 2-5 loss			WT
TK9-0-1	Ex.2-3 loss and deletion	576-581	ATGCC [CTTGA] CTATA	WT
TK9-0-2	Ex.2-5 loss			WT
TK9-0-3	Ex.2-3 loss			WT
TK9-0-4	-			WT
TK9-0-5[B]	Ex.4 loss			WT
TK9-0-6	-			Δ E7-9
TK9-0-7	Ex.4 loss			Δ E4
TK9-0-8	Ex.4 loss			WT
TK9-0-9	Ex.4 loss			WT
TK9-0-10[C]				WT
TK1001	Ex.2-3 loss			Δ E3
TK1002	Ex.4 loss			WT
TK1003	Deletion	162-164	GTGAT [GAA] GGAGA	WT
TK1004	Ex.2-3 loss			WT
TK1005	Intact cDNA			WT
TK1006	Ex.8 loss			WT
TK1007	Ex.2-6 loss			Δ E3
TK1008	Ex.2-3 loss			WT
TK1009	Ex.4 loss			WT
TK10010[C]	Ex.4 loss			WT

Legend for tables 28 and 29: (-) - indicates absence of RT-PCR product. Mutants labeled 'A', 'B', and 'C' - carried large deletions (as only or one of the PCR products) not confirmed by the absence of corresponding sequence in genomic DNA. 'A' - 109-647, 109-647, TGTTT [ATTCC.. ..AAAAG] CAAAA ; 'B' - 37-658, AGTGAT [GATGAA.... ...GCCTAA] GATGAG; 'C' - 45-566, GAACC [AGGTTATGACCTTGA.. ..GTTGT] AGGATATGCCCTTGA;

Table 29. *Hprt* cDNA mutations recovered in Etoposide-treated TK6 cultures

MUTANT	MUTATION	POSITION	SEQUENCE CONTEXT
TK7-125-1	Ex.2-3 loss		
TK7-125-2	Ex.4-8 loss		
TK7-125-3	No cDNA		
TK7-125-4 [C]			
TK7-125-5	Ex.2-3 loss		
TK7-125-6	Ex.2-3 loss		
TK7-125-7	Ex.2-3 and 5 loss		
TK7-125-8	Ex. 2-8 loss and insertion		
TK7-125-9 [C]	Intact DNA		
TK7-125-10	No cDNA		
TK8-125-1	No mutation found		
TK8-125-2	G –> A	88	ATGCT..a..AGGAT
TK8-125-3	Ex. 2-3 loss		
TK8-125-4	Ex.4-8 loss		
TK8-125-5	Ex.2-3 loss		
TK8-125-6	Ex.7 loss		
TK8-125-7	Ex.2-6 loss		
TK8-125-8	Ex.2-6 loss		
TK8-125-9	Ex.2-3 loss		
TK8-125-10	Intact cDNA		
TK91251	Ex.2-3 loss		
TK91253	No cDNA		
TK91254	Ex.2-3 loss		
TK91255	Ex.3-8 loss		
TK91256	Ex.2-5 loss		
TK91257	Deletion	82-84	ATCAT [TAT] GCTGA
TK91258 [C]	Ex.4 loss		
TK91259	No cDNA		
TK912510	Ex.2-5 loss		
TK101251	No cDNA		
TK101252	Deletion	162-164	GTGAT [GAA] GGAGA
TK101253	G –> A	176	GATG ..a.. AGGCC
TK101254	Ex.2-3 loss		
TK101255	Deletion	162-164	GTGAT [GAA] GGAGA
TK101256	Ex.2-5 loss		
TK101257	No cDNA		
TK101259	Ex.2-3 loss		
TK1012510 [C]	Ex.2-4 loss		

Table 29. continued

MUTANT	MUTATION	POSITION	SEQUENCE CONTEXT	mPCR
TK711	Ex.2-3 loss			Δ E2-3
TK712	Ex. 2-6 loss			WT
TK713	Ex. 2-6 loss			WT
TK714	-			2add.bands
TK715	Ex.2-3 and 5 loss			WT
TK716	Deletion	592-615	ATGAA [TACTT.. ..ATGTT] TGTGT	WT
TK717	Ex.4-8 loss			Δ E7-8
TK718	Ex.2-3 loss			WT
TK719	-			Δ E7-8
TK7110[C]				WT
TK8-1-1	Ex.2-7 loss			Δ E7-8
TK8-1-2	Ex.2-3 loss and 4 bp deletion	373-376	CAACT [TTAA] CTGGA	WT
TK8-1-3	-			WT
TK8-1-4	Ex.2-6 loss			WT
TK815	-			Δ E3
TK8-1-6	Ex.2-3 loss			WT
TK8-1-7	Multiple G→A			WT
TK8-1-8	Ex.2-3 loss			Δ E2
TK8-1-9	Ex. 2-6 loss and insertion		Ex.1 - AATTGG.. ..TTGCAG-Ex.7	WT
TK8-1-10	Ex.2-6 loss			WT
TK911	Ex.2-3 loss			WT
TK912	Ex.2-6,8/ Ex.2-4 loss			Δ E3
TK913	Ex.2-8 loss			Δ E3
TK914	-			WT
TK915	Ex.2-3 loss and deletion	576-581	ATGCC [CTTGA] CTATA	WT
TK916	-			Δ E1-9
TK917	Ex.2-3 loss and deletion	576-581	ATGCC [CTTGA] CTATA	WT
TK918	Ex.2-3 loss			WT
TK919	Ex.2-6 loss			WT
TK9110	Ex.7-8 loss			Δ E7-8
TK1011	Deletion	162-164	GTGAT [GAA] GGAGA	WT
TK1012	-			WT
TK1013				WT
TK1014	Ex.2-3 loss			WT
TK1015	Ex.2-6 loss			WT
TK1016[C]	Deletion	162-164	GTGAT [GAA] GGAGA	WT
TK1017	Ex.4-8 loss			Δ E7-8
TK1019	-			Δ E6-9
TK10110	No mutation found			WT

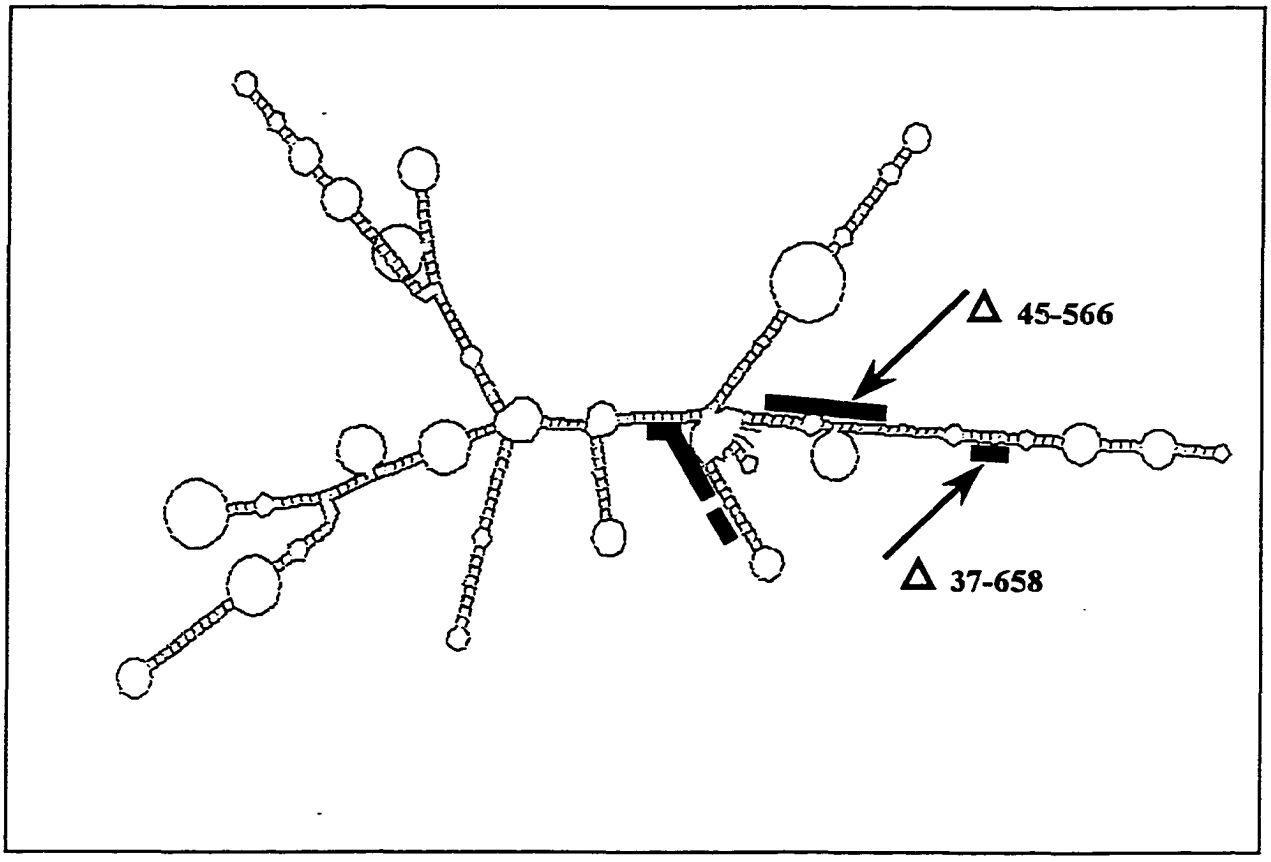

Figure 15. Relative position of direct repeats flanking large cDNA deletions in mutants with the normal cDNA pattern
Legend: Image is obtained by using RNADRAW program.

EXON 1 cgg▲ct▲ccgttATGGCGA▲CCCGCAGC▲CCTGGCGT▲CGTGgtgag▲c

EXON 2 tatttctttttcagATTAGTGATGATGAACCAGGTTATGACCTTGATTTATTTTGCA▼TA▲CCTAATCATTATGC▼TGAG
GATTTGGAAAGGGTGTTTATTCCTCATGGACTAATTATGGACAGgtaagt

EXON 3 ttatttc▼tgtagGACTGAACGTCTTGC▼TCGAGATGTGATGAAGGAGATGGGAGG▲CCATCACATTGTAGC▲C▼CT
CTGTG▼TG▲C▼TCAAGGGGGG▲CTATAAATTCTTTGCTGA▲CC▼TGCTGGATTA▲CATCAAAGCACTGAATAGAAATAGT
GATAGATCCATTC▼CTATGAC▼TGTAGATTTTATCAGAC▼TGAAGAGC▼TATTGTgtgagta▼tat

EXON 4 ttttttttaactagAATGACCAGTC▼AA▲CAGGGGA▲CATAAAAGTAATTGGTGGAGATGATCT▲CTCAACTTTAACT
GGAAAGgta▼tgt

EXON 5 tctttttcttctagAATGTCTTGATTGTGGAAgtaagttca▲cat

EXON 6 t▲ctttttgaaagGATATAATTGAC▼ACTGGCAAAACAATGCAGA▲CTTTGCTTT▲C▼CTTGGTCAGGCAGTATA
ATC▼CAAAGATGGTCAAGGTC▼G▲CAAGgta▼tgt

EXON 7 tttgtaattaacagCTTG▲C▼TGGTGAAAAGGAC◆CCCACGAAGTGTTGGATATAAGCCAGACTgtaagt

EXON 8 atgattctttttagTTGTTGGATTTGAAATTCCAGACAAGTTTGTTGTAGGATA▼TGC▼CCTTGACTATAATG
AATA▲C▼TTCAGGGATTTGAATgtaagt

EXON 9 atttttttttatagCA▼TGTTTGTGTC▼ATTAGTGAAACTGGAAAAGCAAAATACAAAGCC▼TAAgatgagagtt▲c

Figure 16. Potential topoisomerase II cleavage sites in hprt splice sites and coding sequences
Legend: Potential cleavage sites were determined using DNASTAR application. Consensus sequences C▼nnnnG and (Pur▼Pir)n were taken from Pommier et al., 1991; and Spitzner et al., 1994. Symbols ▼ and ▲ indicate location of sequences on upper or lower strands. Deletions underlined.

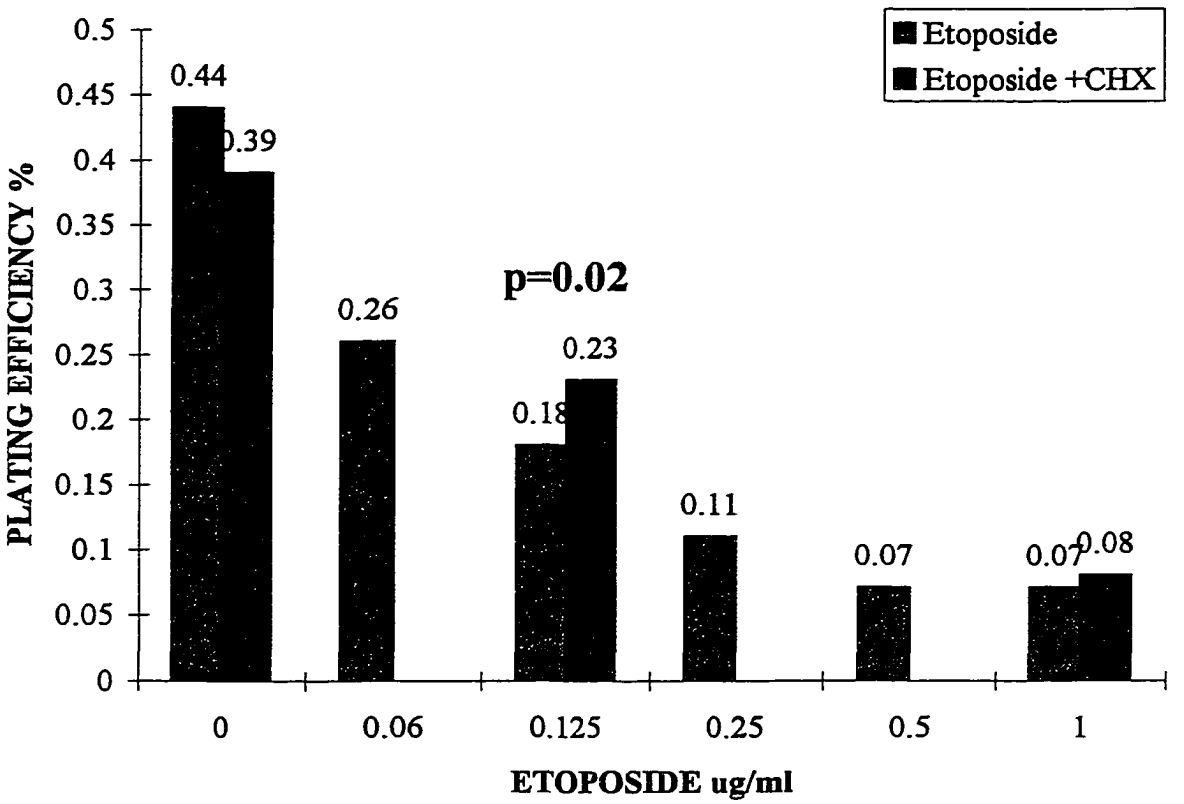

Figure 17. Etoposide cytotoxicity in TK6 cells in presence of cycloheximide.

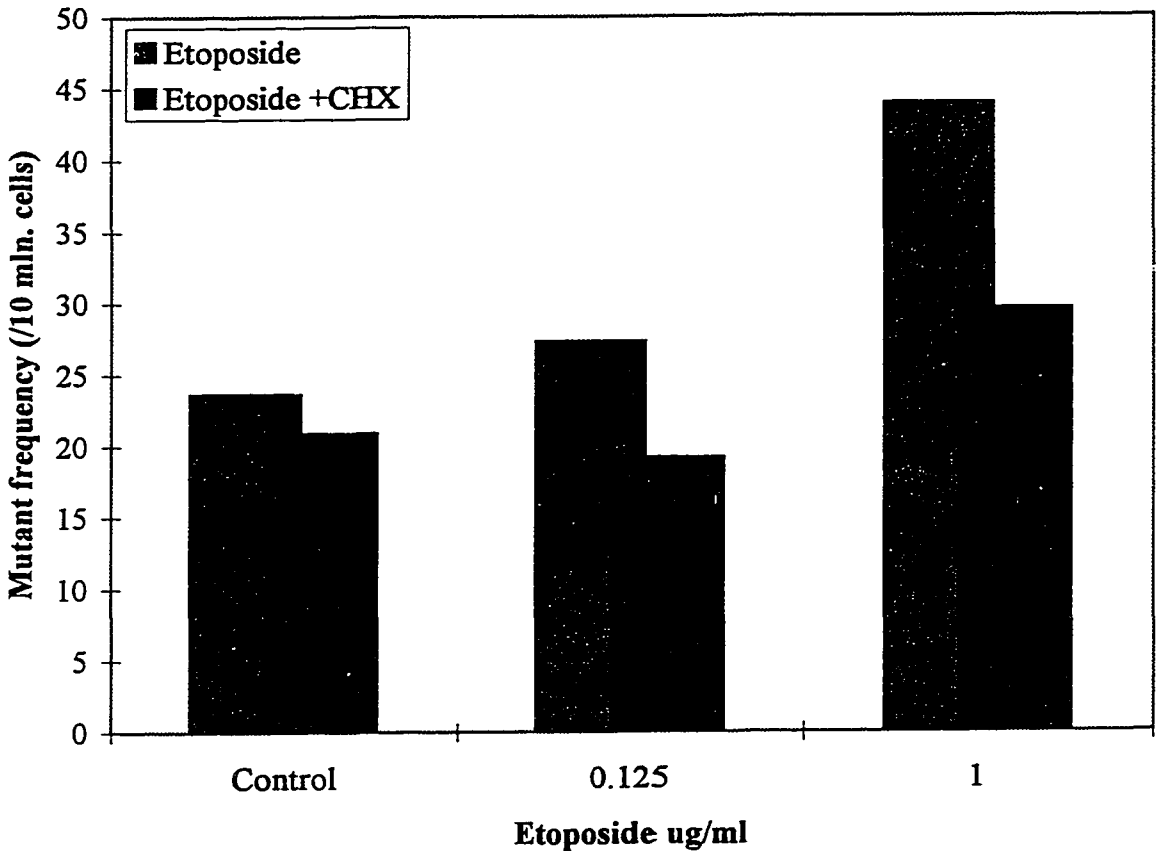

Figure 18. Etoposide mutagenicity in TK6 cells in presence of cycloheximide.

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