IARC Scientific Publications No. 142
International Agency for Research on Cancer, Lyon, 1997

Somatic cell mutations in cancer epidemiology

gy Unit, Flesearch School of
irth Building, University of Leeds,

R.J. Albertini and R.B. Hayes

Somatic cell mutations arising *in vivo* in reporter genes and in cancer-associated genes may now be measured in humans. Background mutation levels and mutational responses following various mutagen exposures are reviewed in this chapter. The detection methods are compared for similarities and differences based on the underlying biology of the systems. Currently available data on molecular mutational spectra are reviewed and the utility of such information is discussed in terms of mutagen exposure characterization and for defining the mutagenic basis of carcinogenesis. In addition to the reporter gene assays, recently developed assays for mutation in cancer-associated genes are considered. The strengths and limitations of using somatic cell mutations for cancer epidemiology and areas for future research are discussed.

Somatic cell mutations occur regularly and universally in all humans. Some are 'naturally occurring', arising continuously as spontaneous replication errors or in response to endogenous mutagens or DNA metabolism, while others are induced by external mutagens which are ubiquitous in the environment. Mutations are central to human carcinogenesis, and, consequently, exposures that cause mutations are also suspect of causing cancer. In principle, subjects who have experienced greater exposure to genotoxic agents will accumulate greater numbers of mutations, so that quantitation of the frequency of mutational events serves to identify heavily exposed individuals. In addition, specific genotoxic agents may produce specific types of mutations, implying that characterization of mutational errors (mutational spectrum) will be useful for identifying exposure to specific agents.

Modern in-vivo somatic mutation studies may employ any of seven assays for measuring in-vivo mutations in five different reporter genes, i.e. not functionally related to carcinogenesis. There are also methods for detecting mutations in cancer genes (oncogenes and tumour suppressor genes). Extensive databases are available for two of the reporter gene assays (*GPA* and *hprt*), while limited information is available for all others. Here we describe the genetic bases and methods for the major assays, summarize the results of applications in humans, and discuss the uses and limitations of the methods for future epidemiological studies.

Reporter gene somatic cell mutation assays: genetic basis and methods

The haemoglobin (Hb) genes

Genetic basis (Table 1). Hb is a tetrameric protein that constitutes >99% of the protein in non-nucleated mature red blood cells (RBCs) (reviewed in Stamatoyannapoulos et al., 1984). Several polypeptide chains, encoded by genes at several linked loci, constitute the intact Hb molecule, depending on the stage of development of the individual. Following birth, adult HbA ($\alpha_2\beta_2$) constitutes >95% of the Hb in mature RBCs, with a small amount of HbA2 ($\alpha_2\delta_2$) also present. The ε,γ (two loci) δ and β Hb genes are on chromosome 11p; the ζ and α (two loci) Hb genes are on chromosome 16p (Deisseroth et al., 1977, 1978).

Three classes of mutations affect the Hb genes. Two, the thalassaemias and hereditary persistence of fetal Hb (HPFH), produce abnormal levels of Hb polypeptides. These are not useful for mutational studies however, as non-genetic influences can reduce Hb levels in RBCs (Stamatoyannapoulos *et al.*, 1984). The third class of Hb mutations produces structural alterations in Hb. Over 500 such changes have been described, most being single amino acid changes in the α or the β chain. Of these, the best known is an A to T transversion in the gene that gives 'sickle cell' haemoglobin, or HbS. Although the gene is autosomal and contains three exons spanning 2 kb, the effective mutational target size for single base changes is a

Table 1. Somatic reporter gene mutations in humans: the reporter genes

Cells	Gene(s)	Chromosome	Gene size	Target size
RBC	Hb gene	Autosome, 11p	3 exons, 2 kb	bp (small)
	Hb gene	Autosome, 16p	3 exons, 2 kb	bp (small)
RBC	*GPA*	Autosome, 4q	7 exons, 44 kb	>44 kb (large)
T-lymphocytes	*HPRT*	X-linked, Xq	9 exons, 44 kb	>44 kb (large)
T-lymphocytes	*HLA-A*	Autosome, 6p	7 exons, 5 kb	>5 kb (large)
T-lymphocytes	TCR	Autosome, 14q	Large 'multi-gene'	Probably large
	TCR	Autosome, 7q	Large 'multi-gene'	Probably large

single base pair. The assay is thus limited to the detection of point mutations, which limits its practical sensitivity and restricts to a narrow range the spectrum of mutational mechanisms detected.

Hb variant frequency (VF) assays (Table 2). G.P. Stamatoyannopoulos and co-workers originally developed an assay based on highly specific antibodies that discriminate the different mutant haemoglobins from each other and from normal haemoglobin, requiring samples of 1 ml blood (reviewed in Stamatoyannopoulos et al., 1984). Scoring techniques for rare variant cells with a mutated Hb are based on fluorescence labelling of fixed RBCs on slides. As haemoglobin is an intracellular protein, fixation and permeabilization are required for antibody entrance. Recent refinements have included newer methods for permeabilizing and labelling RBCs in suspension for automated cell sorting and counting (Bigbee et al., 1981). More recently, automated microscopy has been used to screen large numbers of cells on slides (Tates et al., 1989). One or more structural Hb mutations may be scored in an assay.

Although there have been no systematic descriptions of all technical sources of error in this assay, quality of RBCs and extent of permeabilization could be major sources, as could the quality of the immunological reagents and non-specific staining. Poisson counting of rare events produces relatively large errors.

The GPA gene

Genetic basis (Table 1) Glycophorin-A (GPA) is a polymorphic glycoprotein on RBC surfaces present at approximately 5×10^5 molecules/cell (Furthmayer, 1977; Gahmberg *et al.*, 1979). The *GPA* gene on chromosome 4q spans 44 kb and contains seven exons (Kudo & Fukuda, 1989). In principle, it is a large target for mutation. It has two codominantly expressed (M and N) alleles (Furthmayer, 1978) (except for rare nulls) which have approximately equal frequencies in all populations (Cartron *et al.*, 1990). Thus, approximately 50% of humans are M/N heterozygotes with both forms of the glycoprotein on RBC surfaces. The M and the N molecules differ by two non-adjacent amino acids and cannot be interconverted by simple point mutations.

GPA VF assays (Table 2). The currently used *GPA* assay measures the frequency of variant cells that have lost expression of the M form in blood samples from heterozygous individuals (M/N) (Langlois *et al.*, 1990; Jensen & Bigbee, 1996). A small (<1 ml) sample of blood is treated soon after blood draw to fix spherical erythrocytes which are then kept at 4°C until analysis. Variant cells are detected by flow cytometry with distinguishable fluorescent-labelled monoclonal antibodies specific for the N and M forms and counting variant cells that bind the anti-N but not the anti-M antibody. Simple loss mutations, many representing 'point mutations', are expressed as rare 0/N cells (Grant & Bigbee, 1993). These are referred to as 'hemizygous variants'. More complex mutations are expressed as rare N/N cells on the M/N background, i.e. a loss of function of one allele with double expression of the other. These are thought to arise from mutational events that lead to cellular homozygosity (or loss or

Table 2. So

Gene and cell	Sample size
Hb in RBC	1 ml
GPA in RBC	1 ml
HPRT in T-cells	10–50 ml
HLA in T-cells	10–50 ml
	5–10 ml
TCR in T-cells	Few ml

Table 2. Somatic reporter gene mutations in humans: the assays

Gene and cell	Sample size	Method(s)	Time for assay	Potential for error
Hb in RBC	1 ml	Immunological staining	Month	RBC quality
		—Manual slides	Days	Permeabilization
		—Automated slides	Hours–days	Immunological reagents
		—Cytometry		Non-specific staining
				Poisson counting
GPA in RBC	1 ml	Immunological staining	Hours–days	RBC quality
		—Cytometry		Immunological reagents
				Non-specific staining
				Poisson counting
HPRT in T-cells	10–50 ml	Short-term		Cycling cells give phenocopies
		Autoradiography		Observer error
		Manual slides	Days–week	Cell culture factors
		Automated slides (potential)		
		BrdU incorporation with differential fluorescence		Non-specific staining changes
		Manual slides	Days–week	Poisson counting
		Automated slides	Days	Instrumentation for automated assays
		Cytometry (potential)	(Short)	Poisson counting
		Cloning	Weeks	Inverse correlations between CE and MF
		Selection in tissue culture		Observer error
				Reagents and culture conditions
				Poisson counting
HLA in T-cells	10–50 ml	Cloning	Weeks	Non-specific lack of immunocytotoxicity
		Immunoselection (cytotoxicity) *in vitro* with cell culture outgrowth		Observer error
				Reagents and culture conditions
				Immunological reagents and complement
				Poisson counting
	5–10 ml	Immunological staining		Non-specific failure to label
		Cytometry		immunological reagents
				Instrumentation
				Poisson counting
TCR in T-cells	Few ml	Immunological staining		Non-specific failure to label
		Cytometry		immunological reagents
				Instrumentation

heterozygosity), such as chromosome missegregation, somatic recombination or gene conversion (Grant & Bigbee, 1993). These variants are termed 'homozygous variants'. The rare 0/N and N/N cells that have lost expression of the M allele fall within prescribed areas of the cytogram. The variant frequency (VF) is defined as:

$$VF = \frac{\text{number of 0/N or N/N variant erythrocytes}}{\text{total number of red blood cells analysed}}$$

The quality and age of the RBCs in the blood sample can affect results, necessitating the rapid fixation of collected samples. Antibody lot, quality and preparation are potential sources of technical variability, as are changes in cytometer performance. Poisson counting of small numbers produces relatively large errors. A gold standard has recently been developed for the *GPA* assay (Jensen & Bigbee, 1996). Cryopreservation of formalin-fixed spherical RBCs in medium and DMSO, and storage at –80°C or –150°C maintain samples with stable VFs for up to 6 months. Reference standards are available for interlaboratory quality control and standardization.

The HPRT gene

Genetic basis (Table 1). The *HPRT* gene encodes the HPRT enzyme which is constitutively expressed but dispensable in virtually all mammalian cells. HPRT phosphoribosylates its normal substrates hypoxanthine and guanine for conversion to inosinic acid (Stout & Caskey, 1985) and is required to phosphoribosylate purine analogues such as 6-thioguanine (TG) to their cytotoxic forms (Albertini, 1985a ; Stout & Caskey, 1985). Cells with normal HPRT activity are susceptible to the cytotoxicity of TG and related agents; mutants are resistant, thus providing a basis for selection.

The *HPRT* gene is X-linked and expressed as a single copy in all cells. The gene spans 44 kb and contains nine exons (Patel *et al.*, 1984). A total of 55 kb of DNA including and surrounding this gene has been sequenced, making the region one of the best characterized for mutational studies. In principle, *HPRT* is a large target for mutation.

HPRT VF and mutant frequency (MF) assays (Table 2). There are two assays for assessing *in vivo HPRT* mutations in human T-lymphocytes. Both are based on the resistance of mutant cells to HPRT-dependent cytotoxicity of the purine analogue TG (Albertini *et al.*, 1990). Both assays require similar collection and fractionation protocols. Although the assays can be performed on 5–10 ml of blood, it is preferable to obtain 30 ml or more.

The VF assay (Table 2). The first assay to be developed was a short-term phenotypic assay which has the advantage of speed and potential for automation but the disadvantage of consuming the TG^r T-cells which are then not available for study (Strauss & Albertini, 1977, 1979; Stark *et al.*, 1984). As there is no way to unequivocally demonstrate the mutational basis of the TG^r cells, they are termed variants and their frequencies in blood are termed VFs. For assay, cryopreserved MNCs are thawed and stimulated with PHA in replicate short-term cultures, with or without TG, and incubated until culture termination at 24–30 h (cryopreservation is required to avoid labelling of normal T-cells that are in 'in cycle' *in vivo*; Albertini *et al.*, 1981). At termination, fixed cells are added in measured volumes to microscope slides, and then stained, autoradiographed and scored. Recent protocol modifications use BrdU staining and scoring by differential fluorescence (Ostrosky-Wegman *et al.*, 1988), approaches suitable for automation using either cell cytometry or image analysers.

There are several technical sources of variability in the short-term autoradiographic assay. Only rare labelled cells are counted, so Poisson errors inherent in counting small numbers can be large. The slide-based method is laborious and susceptible to observer error. Automated assays will have concerns with instrumentation. T-cells that are cycling *in vivo* may become labelled and be scored as mutants, unless measures such as cryopreservation are employed to remove this effect (Albertini *et al.*, 1981).

The MF assay (Table 2). The second *HPRT* assay depends on direct *in vitro* cloning in TG and, although laborious, allows for mutant isolation, in-vitro propagation and molecular analyses (Albertini *et al.*, 1982; Morley *et al.*, 1985; Henderson *et al.*, 1986; O'Neill, *et al.*, 1987). Fresh or thawed, cryopreserved MNCs are washed and plated in culture medium for direct cloning in the presence or absence of TG. (Cryopreservation is not required here to remove 'phenocopies' as it is in the autoradiographic assay, but it is convenient.)

A mutant frequency (MF) (and its confidence interval) is calculated from the ratio of the cloning efficiency of T-cells in the presence of TG to the cloning efficiency in its absence. There are large methodological differences between the two kinds of *HPRT* assays, which may give somewhat different results.

There are several sources of technical variability in the cloning assay (Robinson *et al.*, 1993). All investigators have reported a strong inverse correlation between control or non-selected cloning efficiencies and calculated mutant frequencies; observer error with failure to recognize slow-growing mutant colonies may result in variability. Attention to reagents and conditions is critical. Fortunately, the ability to cryopreserve MNCs for subsequent testing provides a protocol standard to control for intralaboratory drift.

The HLA gene

Genetic basis (Table 1). The several linked *HLA* loci include two classes of genes that encode cell surface recognition or restriction molecules of importance for antigen presentation in immune responses (Bodmer, 1984; Janeway & Travers, 1994). These genes constitute the major histocompatibility complex (MHC) in humans. Although the loci are extremely polymorphic, some alleles are present in a high proportion of individuals, i.e. approximately 50% of the population is heterozygous for either the *HLA-A2* or the *HLA-A3* allele.

The *HLA* complex is on chromosome 6p. In practice, mutation studies have been confined to the *HLA-A* gene for primary detection, although molecular studies have defined loss of other linked genes. The *HLA-A* gene is autosomal, spans 5 kb and contains seven exons; it is a large target for mutation.

HLA mutant frequency and variant frequency assays (Table 2). There are also two assays for assessing *HLA* mutations in human T-cells. The constitutional *HLA* genetic background of an individual must be known to measure somatic mutations at this locus, i.e. an individual must normally express one of the test HLA antigens in heterozygous form. The assays measure cellular loss of one codominantly expressed antigen.

Cloning (MF) assay (Table 2). In the cloning assay, (Janatipour *et al.*, 1988; McCarron *et al.*, 1989), peripheral blood is obtained and the MNC fraction is separated. As for *HPRT*, this assay involves inoculation of MNCs into the wells of microtitre plates in limiting dilutions. Selection is due to cytotoxicity by a relevant antibody in the presence of complement. Following selection, cells are directly inoculated into microtitre plates essentially as in the *HPRT* cloning assay. Colonies are scored at 16–20 days by inverted phase microscopy.

One major source of technical variability in the *HLA* assay is lack of killing by specific antibody. However, these 'phenocopies' can be recognized by testing growing colonies for resistance to the specific selecting antibody. The other sources of technical variability are inherent in cell culturing. The sources of immunological reagents and complement and the conditions of immunoselection can also produce technical variation. It should be possible to produce a cryopreserved cell standard as described for *HPRT*.

VF assay (Table 2). A short-term flow cytometry assay has also been described as measuring *HLA* loss mutations in T-cells (Kushiro *et al.*, 1992). The method uses specific biotin-labelled anti-HLA antibodies to label *HLA* gene products on T-cells, FITC-conjugated monoclonal anti-CD3 antibody to label all T-cells, and two-colour cytometric analysis to score CD3+ T-cells lacking the target HLA antigens.

The rare cells that have lost the target HLA antigen, i.e. less than 1/25 × that of normal HLA+ cells, fall within a prescribed area of the cytogram. The variant frequency is defined as:

$$VF = \frac{\text{number of CD3+ target HLA antigen-lacking lymphocytes}}{\text{total of CD3+ lymphocytes}}$$

Sources of error in the assay are non-specific failure to label cells, immunological reagents, instrumentation and Poisson sampling. When used only in the cytometry mode, the assay may be subject to serious phenocopy errors. However, cell sorting can and has been employed to clone and propagate single variant cells *in vitro* for molecular analyses. In principle, the genetic basis of all variants detected in this assay could be verified by molecular analyses.

The TCR gene

Genetic basis (Table 1). There are at least four T-cell receptor (TCR) genes, i.e. the α, the β, the γ and the

δ genes, located on chromosomes 14q (α and, within it, δ), 7q (β) and 7p (γ). Germ-line TCR genetic segments consist of variable (V), joining (J), diversity (D) (β and γ TCR genes only) and constant (C) regions (Janeway & Travers, 1994). Rearrangements occur during the differentiation of T-cells and are mediated by a recombinase system, termed V(D)J recombinase, through which the different V, D and J regions of any TCR germ-line gene are joined in all possible combinations. This confers the TCR gene uniqueness found in mature T-cells. The rearranged genes characterize and identify a specifically reactive T-cell and its clonal descendants.

The complete TCR on the T-cell surface consists of a constant molecule, termed CD3, and the TCR heterodimeric molecule consisting of either an α and β or a γ and δ polypeptide chain, encoded by the respective TCR genes (Clevers *et al.*, 1988; Kyoizumi *et al.*, 1990). A given T-cell expresses either the α/β heterodimeric TCR or the γ/δ heterodimeric TCR—never both. More than 90% of the peripheral blood T-cells express the α/β TCR. The TCR gene mutational assay currently in use focuses only on CD4+/TCRα/βT-cells.

If one of the molecules of the TCR heterodimer is defective for any reason, the CD3TCRα/βTCR complex fails to form on the T-cell surface, and the CD3 molecule accumulates in the cytoplasm. Allelic exclusion operates for the TCR genes, rendering any cell functionally hemizygous for gene expression. Therefore, mutational loss of gene function is not masked by a second allele.

TCR variant T-cells are recognized by the absence of the CD3 molecule from the surface of a CD4+ T-cell. The presumed mutation arises in one of the TCR genes (α or β). (As allelic exclusion is not operative for the CD3 gene, mutational loss of function would require somatic mutation of both alleles at this locus.)

The TCR genes are large genetic segments when in the germline configuration. However, somatic mutations may arise in rearranged genes, which are much smaller. It is not possible at this time to define a target size for the TCR gene mutations.

The TCR VF assay (Table 2). The TCR gene mutation assay is also based on analysis by flow cytometer (Kyoizumi *et al.*, 1992). Commercially available anti-CD3 is phycoerythrin-labelled and anti-CD4 is FITC-labelled, allowing for double labelling of normal T-cells. Variant cells are CD4+ cells that have lost expression of CD3. VFs are calculated as the number of CD3– cells, i.e. cells with CD3 expression level less than 1/25 × that of normal CD4 cells, divided by the total number of CD4+ T-cells.

Sources of technical variability for the TCR gene mutation assay are variabilities in immunological reagents and in flow cytometer performance. Furthermore, as detection of the cell surface molecules are the scored phenotype, the condition of the cells, surface perturbations, etc. could be potential sources of technical variability. Large numbers of variant cells are scored in this assay, and so errors inherent in counting small numbers should not be a problem. As it is not possible to clone most of the TCR gene variants *in vitro* for further analyses, the mutational basis of the measured phenotype is usually not demonstrable.

Reporter gene somatic mutations

General population studies

There is an extensive literature on in-vivo somatic mutations in humans, with extensive databases for *GPA* and *HPRT* mutations (reviewed in Albertini *et al.*, 1990; Cole & Skopek, 1994). This section presents representative results for in-vivo variant and mutant frequencies and molecular mutational spectra.

Hb variant frequency. The Hb mutation assay has been used in demonstration studies of very few subjects, showing in the original report a mean background VF of 11.0×10^{-8} (range $4–30 \times 10^{-8}$) for the single base changes producing the gene HbS and HbC mutations and, in a more recent report on five subjects, a lower mean background VF value of 3.7×10^{-8} (Stamatoyannopoulos & Nute, 1981; Bernini *et al.*, 1990). Intra-individual VF values have remained stable over relatively long time intervals, i.e. at least months, and smoking reportedly elevates the haemoglobin VF values approximately twofold.

GPA variant frequency. Hundreds to thousands of individuals have been studied for in-vivo *GPA* mutations using different versions of the assay (reviewed in Cole & Skopek, 1994). In general, mean hemizygous and homozygous VF values for adults have each averaged 10×10^{-6}, with the for-

mer somewhat lower and the latter somewhat higher. There is wide interindividual variability in VF. Values for newborns and children have been lower than in adults, indicating a clear age effect, with about a twofold increase by age 70. Smoking shows a weak association with increased VF, but systematic differences are not found by gender or ethnicity.

HPRT variant frequency. For the short-term tests, most *HPRT* T-cell VFs have been determined using the autoradiographic version of the assay. Mean values in normal populations range from 10^{-6} to 10^{-5} (reviewed in Albertini *et al.*, 1990; Cole & Skopek, 1994). VFs are increased in adults compared with newborns, and in adults they generally show an increase in frequency with age. Interindividual variability ranges from 10-fold to 30-fold and smoking has generally been associated with an increase in variants. Repeat sampling from individual donors has shown differences of twofold to fourfold in most instances, although differences as great as ninefold have been reported.

HPRT mutant frequency. The largest in-vivo somatic mutation database is for *HPRT* in T-cells, as determined by cloning assay (reviewed in Albertini *et al.*, 1990; Cole & Skopek, 1994). As these mutations are being confirmed by molecular studies, frequencies of TG^r cells are termed MF. A recent statistical analysis of four large data sets (Sussex, UK; Vermont, USA; Paris, France; and Leiden, the Netherlands), including 72 newborns, 70 children and 418 adults, showed ranges of MF values of $0.1–14.7 \times 10^{-6}$ for newborns, $0.5–39.5 \times 10^{-6}$ for children, and $0.8–81.7 \times 10^{-6}$ for adults, with a consistent trend from birth to old age (Robinson *et al.*, 1994). Smoking was clearly associated with increased MFs in the Sussex and Leiden data sets only, although a recent analysis in Vermont also showed a smoking effect. Neither sex nor ethnicity has been found to influence *HPRT* MFs.

HLA mutant frequency. As *HLA* mutations determined by cloning can be confirmed by molecular studies, frequencies of antigen loss cells are also termed mutant frequencies. Mean *HLA* MF values are $2–3 \times 10^{-5}$. *HLA* MFs increase with age, but the effects of tobacco use have not been investigated (reviewed in Albertini *et al.*, 1990; Kyoizumi *et al.*, 1992).

HLA variant frequency. *HLA* VFs determined by cytometric assay are at least five times higher than the clonally determined *HLA* MFs reported earlier, i.e. 1.5×10^{-4} and 0.7×10^{-4} for *HLA-A2* and *HLA-A24* loss variants, respectively (Kushiro *et al.*, 1992). (It is noteworthy that the anti-HLA-A2 antibody was the same for the cloning and cytometric assays.) The reasons for this and for the difference between VFs determined for the two *HLA-A* alleles are unknown.

TCR variant frequency. There are relatively few published reports of background TCR gene VFs. As this is a phenotypic assay, the CD3 loss T-cells are referred to as variants. The background VFs are remarkably high compared with other reporter genes (except *HLA* loss determined by cytometry), i.e. 2.5×10^{-4} (Kyoizumi *et al.*, 1990, 1992). The reasons for this are unknown, although speculation has centred on the role of this genetic region in immune diversification, which may require hypermutability (*HLA* also is involved in immune diversification, but only shows high frequencies of variants in the cytometric assay). Mean TCR VFs increase with age and are reported to be one- to fourfold higher in males. No published reports of studies on newborns or young children, or of smoking effects are available.

Studies in the inherited genetic instability

Marked increases of several VFs and MFs have been observed in patients who are homozygous for certain rare genetic instability syndromes due to defects in DNA repair (reviewed in Albertini *et al.*, 1990; Cole & Skopek, 1994). For example, ataxia telangiectasia (AT) homozygotes have shown increases in *GPA* hemizygous (0/N) and homozygous (N/N) VFs, *HPRT* MFs and TCR VFs, although no effect was seen for Hb VFs. Marked excesses in somatic cell mutations have generally also been found in Bloom's syndrome and Fanconi's anaemia (FA) patients. However, a recent study did not detect increases in *HPRT* MFs but did find *GPA* VF increases in FA (Sala-Trepat *et al.*, 1993). (An earlier study did show *HPRT* MF increases in FA; Vijayalaxmi *et al.*, 1985). For xeroderma pigmentosum (XP), increases were found for *HPRT* T-lymphocyte MFs, but not for *GPA* or Hb VFs. As the cause of genetic instability in XP is hypersensitivity to UV, this finding supports the occurrence of somatic mutations in T-cells as they circulate

throughout the body—in this case in skin, where they are subject to UV irradiation. By contrast, the RBC precursor cells, in which *GPA* and Hb mutations must occur, do not receive similar UV exposures. Therefore, somatic mutation appears linked to a single body compartment for the RBC precursors, but not for the T-cells.

Studies of environmental exposures to genotoxic agents (reviewed in Cole & Skopek, 1994)

Hb variant frequency. Increased HbS and HbC VFs were found among subjects exposed to X-rays, and Hb_{Leiden} VFs were increased after accidental exposure to ^{137}Cs (Stamatoyannopoulos & Nute, 1981; Bernini *et al.*, 1990). Another study found HbS VFs of 8, 18, 27 and 43 $\times 10^{-8}$ in four individuals following their exposure to ethylene oxide, compared with a background range in unexposed individuals of 0–8 $\times 10^{-8}$ (Tates *et al.*, 1989). However, the three exposed individuals with the most elevated values were also smokers. There have been too few studies of Hb mutations following mutagen exposures to evaluate its performance.

GPA variant frequency (cytometric assay). By contrast to the Hb system, there is a wealth of data on *GPA* mutations following human mutagen exposures.

Radiation. Among the earliest population studies with the *GPA* system were those of atomic bomb survivors. Significant *GPA* mutation inductions with radiation exposures were reported in two studies, with the latter showing 63.0 $\times 10^{-6}$/Gy, 32.0 $\times 10^{-6}$/Gy and 0.14 $\times 10^{-6}$/Gy for 0/N, 0/M and M/M variants, respectively (Langlois *et al.*, 1987; Kyoizumi *et al.*, 1989). (These were performed with an earlier version of the *GPA* assay that could also measure loss of the N form of *GPA*.) Similar but somewhat lower values for hemizygous variant increases have been reported more recently (Langlois *et al.*, 1993). All results are population means; in the absence of pre-exposure reference values, individual VFs could not be used to determine individual radiation exposures, as greatly elevated VFs were widely variable in high-dose subjects. These values were obtained more than 40 years after the mutagen exposure, indicating that the *GPA* marker can be long lived. This long memory and the wide interindividual variabilities in response are both consistent with the multipotent bone marrow stem cells being the mutational targets at the time of the bomb blasts. As this stem cell pool contains a limited number of cells, high-dose irradiation reaching it leaves few survivors and wide fluctuations of induced mutants among individuals. Although the numbers of variants among individuals will have an enormous range, the mean VFs in the population at the different radiation exposure levels will reflect the dose. Other studies, in Goiana, Brazil, and among victims of the Chernobyl accident, have reported similar results (Straume *et al.*, 1991; Jensen *et al.*, 1995).

By contrast to the atomic bomb results, patients receiving local irradiation to solid tumours (Hodgkin's disease, prostate) have shown no elevations of *GPA* VFs (Mendelsohn, 1990; Grant & Bigbee, 1994). The geometry of radiation exposures, as well as intensity and duration, appear to be important in determining the *GPA* mutational response. This result is consistent with the lack of VF elevations in XP patients noted above, in that both point to the bone marrow as the sole site of *in vivo* mutations. However, the results of a study in Japanese patients who had received the emitter Thorotrast (^{232}Th) in the 1930s and 1940s for radiographic visualization are somewhat at variance with this (Umeki *et al.*, 1991; Kyoizumi *et al.*, 1992). Although this agent accumulates in body tissues, there were no significant elevations of *GPA* VFs in 10 patients who, as a group, did show elevations of TCR gene T-cell VFs (see below). It is possible that the bone marrow received significantly lower doses than did other tissues.

Chemotherapy-related exposures. Earlier studies of a heterogeneous group of 30 cancer patients treated with a variety of cytotoxic agents and a more recent study of breast cancer patients receiving the combination CAF (cyclophosphamide, adriamycin, 5-fluorouracil) showed consistent elevations of 0/N VFs, while patients receiving CMF (methotrexate substituted for adriamycin) showed lower and more variable elevations (Bigbee *et al.*, 1990). The VF elevations in chemotherapy patients treated with these S-phase specific agents were transient with values returning to normal in several months, consistent with the half-life of RBCs (Bigbee *et al.*, 1990; Grant & Bigbee, 1994).

In children, chemotherapy post-treatment elevations were significant. By contrast with the adult studies, however, when plotted against time post-therapy, VFs for both the 0/N and N/N variants have remained significantly elevated for more than 10 years (Hewitt & Mott, 1992; Mott *et al.*, 1994). Radiation alone did not elevate *GPA* VF in the childhood studies.

Lastly, a study of platinum therapy in adult patients with germ cell tumours reported significant elevations of both 0/N and N/N VFs at different time points throughout therapy, with some significant increases persisting up to 6 months post-therapy (Perera *et al.*, 1992a; Grant & Bigbee, 1994) (*HPRT* T-cell mutations by cloning assay were reported as only marginally elevated in this study—see below).

Unlike localized radiotherapy, therefore, chemotherapy clearly induces *GPA* mutations, which most probably occur in the differentiated rather than the multipotent RBC precursors depending on the chemotherapeutic agent in question. The life spans of the resultant mutants are therefore some multiple of an RBC's life span, producing memories of months to years, depending on when in differentiation most mutations occur. In children there may be a tendency for mutations to occur in early progenitor cells, with some arising in the multipotent stem cells. The *GPA* memory for chemically induced mutations appears to be longer in children than in adults.

Other exposures. Measurements of *GPA* variants, *HPRT* mutant T-cells and DNA adducts were made in iron foundry workers exposed to polycyclic aromatic hydrocarbons (PAHs), and all results were related to ambient PAH exposure levels determined by personal and area monitoring (Perera *et al.*, 1993, 1994). 0/N hemizygous VFs increased slightly, but not significantly, with ambient exposures, while N/N VFs were unrelated to the exposures. Neither set of VFs correlated with PAH–DNA adducts (the *HPRT* mutations in this study are discussed below).

A study of 24 workers in China heavily exposed to benzene and 23 matched controls showed a significant increase in mean N/N VF (13.9×10^{-6} versus background of 7.4×10^{-6}) but no change in 0/N VFs in benzene-exposed workers, suggesting that benzene induces gene-duplication mutations in bone marrow stem cells, which may be the types of relevant pathogenic events in benzene-induced leukemias (Rothman *et al.*, 1995).

A recent study of reinforced plastics workers in Finland exposed to styrene revealed significantly elevated *GPA* N/N VFs among the most heavily exposed workers, particularly women (Bigbee *et al.*, 1996).

HPRT variant frequency (short term assays): radiation. Elevated *HPRT* VFs have been reported for individuals who received heavy accidental exposures to gamma irradiation from a ^{60}Co source in Mexico, for individuals in Kiev, Ukraine, at the time of the Chernobyl accident, and for subjects exposed to gamma irradiation from the ^{137}Cs source in Goiana, Brazil (Tates *et al.*, 1989; Ostrosky-Wegman *et al.*, 1990).

An early study reported radiation-induced elevations of *HPRT* VFs among 12 cancer patients who received 1.8–2.0 Gy/day localized radiotherapy for totals of 20–60 Gy, with the range of VF values in the treated individuals being higher than even the highest background values in control smokers. VFs returned towards normal, from 5 to 32 weeks post-treatment (Ammenheuser *et al.*, 1991).

Chemotherapy-related exposures. The earliest autoradiographic studies of *in vivo HPRT* mutations in humans were in cancer patients receiving cytotoxic chemotherapies (Albertini, 1985a, 1985b). However, the most detailed time-series study of induced *HPRT* VF elevations involved multiple sclerosis patients receiving i.v. bolus infusions of cyclophosphamide (750 mg/m^2 per month) (Ammenheuser *et al.*, 1988). Non-smoking and smoking patients were reported to have mean VFs before treatments of 1.52×10^{-6} and 6.56×10^{-6}, respectively. Two weeks after the first treatment, mean VFs were 29.07×10^{-6} ($n = 4$). All treated individuals showed elevated VFs, the lowest being 11.61×10^{-6}. At 4 weeks after the first treatment, the mean value fell to 5.58×10^{-6} ($n = 4$), and declined further to 3.50×10^{-6} ($n = 5$) at 7–13 weeks after the last treatment. Although clearly indicating a mutagenic effect, these rapid rises and falls in VFs suggest that cytotoxicity, cell division and its abatement may have had some effects at these high acute doses.

Other exposures. An early study of nurses who dispense chemotherapy showed no VF elevations over background (Albertini *et al.*, 1988). However, all safety practices had been observed. By contrast, another early study (using a version of the autoradiograpahic assay that did not take steps to eliminate phenocopies) did show a statistically significant (five- to sixfold) increase in VFs in cyclophosphamide workers (Hüttner *et al.*, 1990).

Several recent occupational studies using the autoradiographic *HPRT* assay have shown increases in VFs at exposure levels considered to be in the acceptable range. Three have involved exposures to butadiene. Thirteen non-smoking workers in a monomer production plant showed mean *HPRT* VFs of 1.0×10^{-6} for the six non-exposed controls, 1.2×10^{-6} for the five low-level exposed workers (0.03 ppm butadiene) and 4.0×10^{-6} for the eight high-level exposed workers (3.5 ppm butadiene). The mean VF of the highly exposed group was significantly elevated compared with the other groups. Furthermore, urinary levels of 1,2-dihydroxy-4- (*N*-acetylcysteinyl)butane, a marker of in-vivo butadiene dose, were significantly elevated in the highly exposed group and correlated significantly with *HPRT* VFs (Ward *et al.*, 1994). A second study of the same worker population and an ongoing study of workers in styrene-butadiene production tend to confirm these results (Ward *et al.*, 1996). Thus, these studies suggest that butadiene exposures at this level have genotoxic effects. (These results using the autoradiographic assay are remarkable because two studies with the cloning assay (in China and the Czech Republic; see below) have failed to show increases in *HPRT* MFs in populations with comparable butadiene exposures.)

The BrdU staining method of short-term assay has been used for only a single study of chemical exposure, i.e. to arsenic in Mexico (Ostrosky-Wegman *et al.*, 1991). Although the *HPRT* VFs in a high-level exposure group were reportedly twice the levels in a low-level exposure group (mean 5.0 vs mean 2.4×10^{-6}), this difference was not statistically significant.

HPRT mutant frequency (cloning assay): radiation. Cloning assays have been used to study *HPRT* mutations in Japanese atomic bomb survivors. All studies have shown low-level MF elevations in exposed individuals, with a 'shallow' dose–response curve (Hakoda *et al.*, 1988; Akiyama *et al.*, 1990; Hirai *et al.*, 1995). This was initially interpreted to indicate that *HPRT* is relatively insensitive to ionizing radiation. It is now realized, however, that the mutational signal has decayed in the 40+ years between the exposure and testing, as a consequence of mutations arising in peripheral T-lymphocytes without renewal from the bone marrow stem cell compartment in adults. In survivors who were young at the time of the exposures, the *HPRT* mutations also arose in precursor bone marrow stem cells—even in multipotent stem cells. This is probably the reason for any elevation at all in mean *HPRT* MF in the exposed populations. Indeed, a molecular study in one survivor showed that at least one mutational event arose in a stem cell before differentiation into the B-, NK and T-cell lineages had occurred (Hakoda *et al.*, 1989).

Several groups have found increased *HPRT* mutational responses to radiotherapy in cancer patients. From an early study of 12 breast cancer patients receiving 2 Gy/day local irradiation sampled shortly after treatment, it was estimated that T-cells circulating through the radiation field would have received 4 Gy irradiation, giving a radiation-induced increase of 6.9×10^{-6} mutants/Gy (Messing & Bradley, 1985). Others have found excesses for patients with breast cancer and Hodgkin's disease, although some investigators report only weak associations (Nicklas *et al.*, 1990, 1991; Sala-Trepat *et al.*, 1990; Branda *et al.*, 1991).

There have also been measurements of *HPRT* T-cell mutations in patients receiving extremely low irradiations (10–15 mGy) during nuclear medicine scans. Initial studies reported significantly increased mean MFs following these low exposure procedures (Seifert *et al.*, 1987), while later investigations did not find such an effect (Bachand *et al.*, 1991; Kelsey *et al.*, 1991; Van Dam *et al.*, 1991). It seems fair to conclude that the extremely low irradiations encountered in nuclear medicine scans are not measurable as mutagenic to *HPRT* and that the differences among studies were due to technical variables.

In contrast to patients, two early studies of radiation therapy and nuclear medicine technicians showed them to have elevated MFs relative to hospital controls based on doses received (2 mSv) in the previous 6 months (Messing *et al.*, 1986, 1989). This same investigative group has recently studied

workers exposed to low-level chronic irradiation (mean 1.34 mSv) in a Quebec factory (Seifert *et al.*, 1993). Although no group differences were found between exposed and non-exposed workers, radiation doses received 48–68 weeks before sampling were significantly positively correlated with MFs. The authors calculated a rate for induction of mutants of 0.7–3.4 × 10^{-4}/Sv, a rate which is similar to, but somewhat less than, that determined earlier for the radiation therapy and nuclear medicine technicians.

Of note, however, is a study of 36 workers at the Sellafield nuclear reprocessing installation, 18 of whom had cumulative recorded radiation doses of <50 mSv and 18 had cumulative doses of 500 mSv accumulated over many years, but which was clearly negative for *HPRT* mutations (Cole *et al.*, 1995).

Chemotherapy-related exposures. Many groups have used the cloning assay to measure *HPRT* MFs in adult patients receiving chemotherapy (Dempsey *et al.*, 1985; Palmer *et al.*, 1988; Sala-Trepat *et al.*, 1990; Branda *et al.*, 1991; Caggana *et al.*, 1991). Increased MFs were found among a group of patients with solid tumours and lymphomas, among breast cancer patients, other malignancies, and among patients treated with low doses of cyclophosphamide for connective tissue disease.

A recent study examined *HPRT* MFs in 15 cancer patients (10 with testicular cancer) and found that cyclophosphamide and ifosfamide were the most mutagenic agents, while adriamycin, 4-epi-adriamycin and bleomycin produced equivocal responses (Tates *et al.*, 1994a). Cisplatin and the etoposide VP16 (a topoisomerase inhibitor) did not increase *HPRT* mutations. These results are in accord with another study of multiple biomarker responses in germ cell tumour patients treated with platinum-based chemotherapeutic regimens, which showed marginal elevation in *HPRT* MFs (but increased *GPA* VFs) (Perera *et al.*, 1992a).

The mutagenicity of chemotherapy in children has been evaluated by the *HPRT* cloning assay. A study of 45 children with acute lymphoblastic leukaemia (ALL), 13 children with acute myelogenous leukaemia (AML) and 28 age-matched healthy controls showed a significantly higher mean MF (7.8 × 10^{-6}) in the treated ALL patients than in the treated AML patients (1.7 × 10^{-6}) and in healthy controls (1.1 × 10^{-6}) (Hirota *et al.*, 1993). Fifteen of the ALL patients had MFs >10 × 10^{-6} and elevations persisted for years. A potentially significant difference between the *HPRT* mutagenic responses induced by chemotherapy in adults and in children is the usually transient nature of the former and the persistence of the latter. This may indicate that, in children, a relatively larger proportion of the mutations are induced in bone marrow stem cells.

Other exposures. Oncology workers, nurses and pharmacists have all shown elevated *HPRT* MFs in different studies, although cloning efficiencies and lymphocyte subpopulations have also occasionally been affected by the exposures, which may have artefactually elevated values (Chrysostomou *et al.*, 1984; Dubeau *et al.*, 1994). Factory workers exposed to nor-nitrogen mustard clearly showed increased *HPRT* MFs compared with controls (Cole & Skopek, 1994).

Two groups of workers chronically exposed to ethylene oxide, i.e. nine hospital workers and 15 factory workers (40 h time-weighted average exposures of 0.25 ppm for the hospital workers and 5 ppm for the factory workers) showed mean MFs for the hospital workers of 12.4 × 10^{-6} and for the factory workers of 13.8 × 10^{-6} (Tates *et al.*, 1991). The latter elevation was significant compared with controls. The sensitivities of the various biomarkers employed in this study for detecting ethylene oxide exposure were haemoglobin adducts > sister chromatid exchanges > chromosome aberrations > micronuclei > *HPRT* mutations. By contrast, acute high-level exposures to ethylene oxide in seven workers had no effect on *HPRT*, SCE or any other biomarker evaluated, leading the authors to conclude that transient exposures to this agent, even at high doses, produce no genotoxic consequences (Tates *et al.*, 1995). In styrene workers, two *HPRT* studies have been negative and one suggestive of an effect in five subjects (Cole *et al.*, 1989; Tates *et al.*, 1994b; Vodicka *et al.*, 1995). In iron foundry factory workers exposed to PAHs at low levels, i.e. from <50 to 200 ng/m^3, *HPRT* mutations did correlate with borderline significance with estimated PAH exposure, but much more significantly with PAH adduct levels (Perera *et al.*, 1993, 1994). (By contrast, *GPA* mutations in this study were not sig-

nificantly elevated and did not correlate with DNA adducts.) In a study of bus maintenance workers, both the *HPRT* MFs and the PAH adduct levels were highest in the most heavily exposed workers, with a highly significant increase in individual MFs observed with increasing adduct levels (Hou *et al.*, 1995b). Two recent studies that used the cloning assay to measure *HPRT* MFs in butadiene-exposed workers have been negative (Hayes *et al.*, 1996; Tates *et al.*, 1996). These butadiene studies contrast with those mentioned above which used the autoradiographic assay.

In summary, ionizing radiation and chemicals at exposure levels found in accidental, household and occupational settings do induce *HPRT* mutational responses. The autoradiographic form of the assay may detect exposures with greater sensitivity than the cloning assay, but this must be demonstrated for the same populations receiving the same exposures for any valid conclusion.

HLA mutant frequency. The cloning assay for *HLA* mutations has not been used, thus far, to study humans exposed to mutagens.

HLA variant frequency (cytometric assay). The cytometric assay for *HLA* mutations was used to study 69 atomic bomb survivors, chosen as low-dose exposed (DS 86 doses = 0 Gy; 1986 estimates) or high-dose exposed (DS 86 doses >1 Gy) (Kushiro *et al.*, 1992). There were no significant increases in VFs with radiation. It was concluded from this study that *HLA* mutations cannot be detected in T-lymphocytes after the 40–50 years' lag between exposure and assay.

TCR variant frequency (cytometric assay). This assay has been used only to study radiation exposures. No significant dose effects were found in a large study of atomic bomb survivors, indicating again that mutational signals in T-cells have decayed in the 40+ years between exposure and test (Kyoizumi *et al.*, 1992). A single individual exposed to 3–4 Gy in Chernobyl was found to have an elevated VF of 21.1×10^{-4} 3.5 years later.

Eighteen thyroid cancer patients treated with ^{131}I from 2 months to 5 years earlier showed a significant linear relationship between the amount of radioactivity administered and the TCR gene VFs (Kyoizumi *et al.*, 1992). The induced mutations per administered dose were calculated as 0.30×10^{-4} TCR variants/GBq. Similarly, TCR VFs in six of 10 patients who received ^{232}Th for radiographic visualizations were elevated compared with concurrent controls (Umeki *et al.*, 1991; Kyoizumi *et al.*, 1992). ^{232}Th remains in the body and produces constant irradiation. By contrast, *GPA* VFs also measured in these patients showed no significant elevations.

Mutation spectra in reporter genes

The T-cell cloning assays allow for molecular analyses of mutations. By far the largest database is for *HPRT*, where mutation events ranging from single base changes to deletions, translocations and recombinations have been identified, and mutational spectra under a variety of circumstances have been described. *HLA* molecular analyses have been limited to Southern blots which have revealed a high frequency of events such as somatic recombination that involve the homologous chromosome. In contrast to autosomal genes, the X-chromosomal *HPRT* gene cannot undergo homologous recombination. However, the mutation spectra defined to date have been for *HPRT* mutations.

HPRT mutational spectra

Thousands of 'spontaneous' *HPRT* mutations arising *in vivo* in human T-cells have now been analysed at the molecular level. There have been several recent reviews of molecular studies, and a computerized database of published results is available (Albertini *et al.*, 1990; Cariello *et al.*, 1992; Cariello & Skopek, 1993; Cole & Skopek, 1994; Cariello, 1994).

The adult background *HPRT* mutational spectrum differs from that in the fetus and in young children. In adults, <15% of mutations arising *in vivo* show gross structural alterations such as deletions, insertions or other rearrangements on Southern blots (Nicklas *et al.*, 1989) (these Southern blot alterations involve >300 base pairs). The remaining 85% of adult *HPRT* mutations have been classified as 'point mutations' and include base substitutions, frame-shifts, smaller deletions and insertions, complex alterations and uncharacterized splice site changes. By contrast, the background *HPRT* mutations in placental cord blood show 75–85% to have gross structural alterations and the remainder to be 'point mutations' (Finette

et al., 1996), although maternal lifestyle factors and exposures may alter this pattern (Manchester *et al.*, 1995). Approximately 35% of fetal mutations show a single kind of DNA gross structural alteration, as discussed below. This reversal of the adult pattern persists in children until approximately the age of five.

The impetus for characterizing background mutation spectra in reporter genes is for comparison with mutational spectra following exposures to specific environmental mutagens. The expectation is that specific mutagens or classes of mutagens will induce characteristic mutational changes. Once identified, these changes can then be used in subsequent population studies to define the nature of exposures. In this sense, it is hoped that reporter gene mutations may serve as restricted biomarkers of exposure by providing specificity for the offending mutagen.

The discovery of induced in-vivo mutational spectra is just beginning. Ionizing radiation produces an *HPRT* mutational spectrum that becomes increasingly dominated by large structural alterations (such as deletions) as radiation doses increase, i.e. ionizing radiation, at least low energy transfer (LET) ionizing radiation, produces deletions at *HPRT* (Nicklas *et al.*, 1990, 1991). Studies of chemical mutagen exposures in humans have thus far given mixed results, probably because of insufficient numbers of mutants analysed. An early report indicated that in-vivo exposures to ethylene oxide induced a G to A transition at G_{197} (Cariello *et al.*, 1992). In-vitro controlled mutagenicity experiments are beginning to show characteristic mutational spectra, e.g. the pesticide malathion may be associated with characteristic deletions in exon 3 (Pluth *et al.*, 1996). Animal studies also show characteristic *HPRT* mutation spectra associated with particular mutagens, e.g. butadiene exposures in mice (Cochrane & Skopek, 1994). Therefore, some degree of mutational specificity from different mutagen exposures has been discovered at *HPRT*.

HLA mutational spectra. (Table 7) Thus far, the *HLA* mutations in human T-cells have been analysed only by Southern blots (Turner *et al.*, 1988; Morley *et al.*, 1990; Grist *et al.*, 1992). However, the investigations have defined loss of the specific target *HLA-A* gene in the mutants and the presence or absence of the *HLA-B* and other linked genes. Approximately 65% of background *HLA* mutants show no change on Southern blots, 2–8% show simple deletions, and 30% show changes compatible with mitotic recombination. Few show gene conversion. The 'no change' and mitotic recombination classes increase significantly with age.

In contrast to background, 75% of the *HLA* mutants induced by ionizing radiation *in vitro* show changes compatible with deletion (Kushiro *et al.*, 1992). Therefore, ionizing radiation is also characterized by deletion mutations at *HLA*.

Cancer-relevant mutational changes 'captured' in reporter genes (Table 3)

The rationale for defining *in vivo* reporter gene mutation spectra was originally to provide 'specificity' for identifying exposures. However, when used as biomarkers of effect, reporter gene mutations must also reflect events with pathogenic significance occurring elsewhere in the genome. To be useful as surrogates for cancer genes, reporter genes must undergo mutagenic processes that have carcinogenic potential.

Both the *GPA* and the *HLA* systems reflect mitotic recombinations among the mutants. This

Table 3. Cancer-relevant mutational changes: 'captured' in reporter genes

GPA	*HLA*	*HPRT*
Mitotic recombination	Mitotic recombination	Large deletions with topoisomerase II breakpoints
Gene conversion	Gene conversion	Fusion genes
Chromosome reduplication	Chromosome reduplication	V(D)J recombinase-mediated recombinations

mutational mechanism is known to underlie loss of heterozygosity (LOH) in several tumour suppressor genes, and, therefore, is an important mutational step in carcinogenesis. Agents that cause such changes in reporter genes, e.g. benzene-induced 'homozygous' *GPA* mutations as noted above, may be human carcinogens (Rothman *et al.*, 1995). Monitoring for such specific mutational changes may be important for predicting individual cancer outcomes.

Although the *HPRT* gene cannot undergo homologous somatic recombination, it does capture a variety of other carcinogenic mutagenic mechanisms. Large deletions and translocations, common in human tumours, are frequent changes in *HPRT* mutations (Nicklas *et al.*, 1989), particularly those following ionizing radiation (Nicklas *et al.*, 1990, 1991). The breakpoints of deletions often occur in DNA sequences with high homology to topoisomerase II consensus cleavage sequences where similar breakpoint sites are seen in the leukemias (Rainville *et al.*, 1995). *HPRT* mutations also may produce fusion genes, another change frequently observed in cancer.

One specific mutational change in *HPRT* is particularly striking because it mimics so well an event seen in virtually all lymphoid malignancies (Finger *et al.*, 1986; Boehm & Rabbitts, 1989; Tycko & Sklar, 1990; Breit *et al.*, 1993). This is the specific intragenic deletion that occurs *in vivo* during fetal life and early childhood, which is the most frequent single class of background *HPRT* mutations during this period of life (Fuscoe *et al.*, 1991; Manchester *et al.*, 1995; Finette *et al.*, 1996). These mutations show all of the characteristics of the V(D)J-mediated recombination that characterizes the TCR gene rearrangements, and they are virtually identical, at the sequence level, to the known cancer-related V(D)J recombinase-mediated mutations in lymphoid malignancies. This mutagenic mechanism with carcinogenic potential is precisely captured in *HPRT*.

As specific mutational mechanisms of carcinogenic significance are recognized, it becomes feasible to develop PCR and other molecular techniques for their rapid identification. Monitoring for these precise events, rather than reporter gene mutations in general, may be the relevant measures in reporter genes when they are used as biomarkers of effect in cancer epidemiology.

Somatic mutations in cancer genes

Several assays have been developed to detect gene mutations in cancer genes or their products.

Cancer-associated 'mutation' assays (Box 1)

Phenotypic 'mutation' assays. Malignant transformations are associated with mutations of oncogenes and tumour suppressor genes. In the case of the former, mutations result in overexpression of a normal protein (or expression of an aberrant protein) essential for cell proliferation. Many oncoproteins and tumour suppressor gene proteins are detectable by immunological techniques, e.g. immunoblot, ELISA, in body fluids such as serum from cancer patients (Brandt-Rauf, 1991, 1992). Studies of banked sera have revealed that, in many patients, the increased oncoprotein levels were present months to years before diagnosis. For environmentally related malignancies, it has been postulated that the causative carcinogen mutated the relevant gene(s) before the onset of clinical cancer. Measurements of these proteins might therefore be used as biomarkers of effect in epidemiological studies to detect cancer-relevant somatic mutations. Although many oncoproteins and tumour suppressor proteins have been studied, attention has focused on the *ras* p21 protein, the extracellular domain (ECD) of the c-*erbB-2* (HER-2, *neu*) p185 protein, the β-transforming growth factor (β-TGF) protein and the p53 protein.

Genotypic 'mutation' assays. Two polymerase chain reaction (PCR)-based molecular assays have been

Box 1. Cancer-associated 'mutation' assays

Phenotypic assays

- Oncogene protein in serum
  - *ras* p21
  - ECD of c-*erb* B-2 p185
  - β-TGF
- Tumour suppressor proteins in serum
  - p53

Genotypic assays

- Hybrid TCR genes
- BCL-2 rearrangements

introduced to detect 'mutations' associated with cancer. Strictly speaking, both measure forms of chromosome aberrations mediated by aberrant V(D)J recombinase activity, as described above. These two assays are described here because they measure directly either a cancer-relevant mutation mechanism or a known cancer-related chromosome translocation.

The first of these assays detects hybrid TCR genes, i.e. Vβ -Jγ or Vγ -Jβ, formed by inversions of chromosome 7 (inv7[p13; q32]) (Lipkowitz *et al.*, 1992). This inversion joins the TCRγ gene on chromosome 7p13-15 to the TCRβ gene on 7q32-35. (This molecular assay may measure events similar to the cytometric TCR gene somatic mutation assay described above, but direct comparisons have not been made.) By chosing primers for PCR from appropriate β and γTCR gene regions, products are formed only when the inversion has occurred. The assay is performed on peripheral blood lymphocytes, presumably the T-cells.

The second molecular method (BCL-2 translocation assay) measures an aberrant V(D)J recombinase-mediated event of direct relevance to cancer (Liu *et al.*, 1994). The chromosome translocation t(14:18) (q32:q21), which occurs at high frequency in non-Hodgkin's lymphomas, brings together the B-cell leukemia/lymphoma-2 (BCL-2) locus on chromosome 18 and the Ig heavy chain joining (J) region on chromosome 14, dysregulating the former and resulting in delayed programmed cell death. Again, by choosing appropriate primers for PCR, products are formed only when the translocation has occurred. The assay is performed on normal peripheral blood lymphocytes, presumably the B-cells.

Cancer-associated gene mutations in humans

The phenotypic assays. Immunological detection of oncogene and tumour suppressor gene products in sera was initially carried out in cancer patients to define the percentage of positives for the different cancers for the different products. As testing moved to serum banks, it became possible to assess sera retrospectively to determine if significant elevations of the serum markers could be found before the onset of cancer. Thus, the utility of these assays was originally for early diagnosis.

Oncogene and tumour suppressor gene proteins have now been used as biomarkers of effect in several human studies. Depending on the methods used and the products studied, these molecules may be found at low levels in healthy control individuals. Therefore, different studies have used different definitions of 'positive'. Furthermore, although some studies measured truly mutant proteins, most have simply measured increased levels of the marker protein, using methods that could not distinguish between mutant and normal molecules. The assumption is that elevated levels of even normal proteins are probably due to somatic mutations and correlate with cancer.

An early study of the *ras* p21 protein in healthy Finnish foundry workers exposed to PAHs in the workplace showed that one of the eight exposed and none of 10 unexposed individuals had detectable serum levels (Brandt-Rauf, 1992). In another study, three of 16 hazardous waste workers exposed to a wide variety of mutagens showed detectable serum *ras* p21 proteins, as did two of 17 unexposed workers (Brandt-Rauf, 1992). Both of the latter, however, were heavy smokers and were only 'trace' positive. One of the exposed workers developed a premalignant colon lesion 1.5 years after testing. Removal of this lesion normalized the serum *ras* p21 protein level.

Serum *ras* p21 proteins were determined in a Polish population study where a doubling in the frequency of high *ras* oncogene expression (>2SD control serum levels) was found in individuals exposed to environmental pollutants (Perera *et al.*, 1992b). A more recent study measured the *ras* p21 serum proteins in butadiene-exposed workers and found no elevations (Anderson *et al.*, 1996).

A study in vinyl chloride (VC) workers monitored the specific mutant Asp13c-Ki-*ras* p21 protein (DeVivo *et al.*, 1994). Four of five exposed workers with liver angiosarcoma and eight of nine with liver angiomas had detectable mutant proteins (by immunoblotting) in their sera. Importantly, 22 of 45 (49%) of the VC-exposed workers with no evidence of liver neoplasia also showed detectable levels. A significant linear trend was found for mutant p21 protein in serum and increasing duration of VC exposure. None of 28 non-exposed individuals had detectable serum mutant p21 protein.

Other oncoproteins have also been studied in mutagen/carcinogen-exposed individuals. Forty-six pneumoconiosis patients (32 asbestos, 10 silicosis) were studied for a variety of oncoproteins

(Brandt-Rauf *et al.*, 1992). Five of 18 with cancer had elevated serum *ras* p21 proteins (total), defined as a fivefold elevation over normal by dilution, compared with only two of the 28 without cancer. There were many pre-diagnosis positive serum values in this study. Pneumoconiosis patients also had significant elevations of platelet-derived growth factor (PDGF) serum proteins.

Another study of multiple serum oncoproteins showed that the β-TGF proteins were elevated in 14 of 33 fire-fighters exposed to a variety of pulmonary mutagens/carcinogens (Ford *et al.*, 1992). No elevations were found in unexposed controls.

Studies are being reported of serum levels of the extracellular domain (ECD) of the c-*erbB-2* protein p185 and the epidermal growth factor receptor (EGF) protein (Brandt-Rauf *et al.*, 1994; Partanen *et al.*, 1994). These are elevated in certain premalignant conditions, in early cancers, and in some exposure situations with a high risk of cancer. Studies of p53 serum protein in early cancers are ongoing. All are being pursued with the goal of developing biomarkers of effect for human mutagenicity monitoring.

The history of the of cancer gene biomarkers is that the original study populations were individuals with cancers. Studies then progressed to individuals with early cancers, then to individuals with premalignant conditions, and finally to individuals exposed to mutagens/carcinogens with a high risk of cancer. This contrasts with the history of the reporter gene mutation assays. In this case, development progressed from studies of healthy individuals exposed to mutagenic agents and were designed to detect exposures. Only recently has attention focused on cancer-relevant mutagenic mechanisms. Thus, development of these two kinds of assays have progressed from opposite directions towards each other, i.e. reporter genes moving from exposure to disease, with the cancer gene mutations moving from disease to exposures.

The genotypic assays. The PCR-based method for detecting the inv7 (p13:q32) chromosome aberration has been used to study normal individuals, patients with AT, and individuals exposed to pesticides (Lipkowitz *et al.*, 1992). Normal background frequencies are 10^{-5}, as determined by limiting dilutions. AT homozygotes have a 100-fold increase in frequency, while heterozygous carriers have normal values. Importantly, individuals heavily exposed to pesticides have had inversion frequencies intermediate between normal control and AT patients.

Standardization of the PCR method for detecting BCL-2 translocation frequencies is just beginning (Liu *et al.*, 1994). Quantification is achieved by a multiple tube method based on Poisson distributions. Individuals with no detectable translocations ($<10^{-6}$) are considered as 'negative.' Twenty-four of 53 blood samples from normal individuals were negative for this translocation. Of the positives, translocation frequencies varied from 0.8 to 32.0×10^{-6}, a 40-fold difference between the lowest and the highest (values $<10^{-6}$ can be detected when frequencies are based on several analyses). When stratified into age groupings of 0–20 years, 21–40 years, 41–60 years and >60 years, these frequencies were 0.29, 0.77, 1.43 and 3.39×10^{-6}, respectively. Variability also increased with age. (It should be noted that, although termed 'translocation frequencies', many of the translocations from specific individuals represent large clones, some of which have persisted for long intervals. Therefore, in these cases the measured frequencies greatly overestimate the translocation events.)

Issues in the application of somatic mutations in epidemiological studies

Strengths and limitations of the methods

At present, seven assays are available for assessing mutations of five reporter genes using two cell types. Mutations scored in RBCs occur in nucleated precursor cells, thus limiting the in-vivo site of mutation to the bone marrow. Moreover, the RBC assays, i.e. Hb and *GPA*, do not allow for molecular analyses of the mutations or the development of direct molecular detection methods. The advantages of the RBC assays is that they require very small blood samples and are rapid, inexpensive and simple. Furthermore, the *GPA* assay, although phenotypic, allows for assessment of mitotic recombination, an important mechanism in LOH. Finally, when mutations arise in the multipotent bone marrow stem cells, they are potentially long-lived and may be used for remote dose reconstructions or nested case–control studies. (However, chemically induced mutations in RBC precursors appear to arise, in part, in more differentiated cells with much shorter persistence, i.e. only months.)

The greatest strength of the lymphocyte assays is that they provide nucleated cells for molecular analyses. Mutation spectra can be determined and direct molecular assay methods developed. Furthermore, as mutations occur in peripheral T-cells, mutations can arise in all body sites. A limitation of T-cells, however, is that tissue culture methods must be used for molecular analyses to be possible. These are usually slow, costly and labour-intensive. In addition, relatively large blood samples must be obtained. The short-term lymphocyte assays circumvent this, using inexpensive and rapid methods, but do not allow for molecular analyses. The mutational memory in the peripheral T-cells is probably a matter of months, at least in adults. Therefore, these mutations will not be of value for remote past exposures.

The time of appearance of mutants in peripheral cells has not been precisely defined for all of the mutation assays. Mutants appear as early as 2 weeks after mutagen exposures, as shown for the *HPRT* autoradiographic assay (Ammenheuser *et al.*, 1988). Usually, however, the time for optimal occurrence will be a period of months. Additional longitudinal studies are needed to establish the time of optimum mutant appearance for various exposures. In any case, mutant cells will usually appear long after the metabolites, SCEs and chromosomal aberrations when these biomarkers are used with somatic mutations in epidemiological studies. The use of multiple biomarkers, however, is usually advantageous in epidemiological studies as some, i.e. DNA adducts, are the best estimators of in-vivo mutagen doses. Several studies have shown correlations of *HPRT* mutations to these biomarkers of exposure but not to ambient exposures.

Assay variability

All of the currently used reporter gene assays show large interindividual variation. Much of this is biological, and epidemiological studies will help to establish the precise causes. It has been shown that the rare genetic instability syndromes are associated with large increases in VFs and MFs. It is not known, however, if lesser deficiencies of DNA repair will also be reflected in detectable increases in mutations. This will require methods to assess more accurately the repair capabilities themselves. An important emerging area of research is the correlation between the various metabolic genotypes and the interindividual differences in somatic mutations. Since these genotypes are associated with different responses for the biomarkers of exposure, i.e. DNA adducts, there is every reason to expect that somatic mutations will also be increased in susceptible individuals. A suggested association between increased *HPRT* MF in individuals of GSTM1 null genotype has recently been reported (Hou *et al.*, 1995a). Eventually, epidemiological studies will have to take all of these into account in assessing somatic mutations.

Physiological, nutritional and pathological changes can also influence interindividual and even intra-individual replicate sample variations in somatic mutations. For example, recent studies have shown that *HPRT* mutations are inversely associated with serum folate levels (Branda *et al.*, 1991). Viral infections may increase the mutability of some somatic cells (Havre *et al.*, 1995). Autoimmune diseases raise in-vivo T-cell *HPRT* MFs, probably as a consequence of increased cell proliferation (Theocharis *et al.*, 1995). Haematopoietic stress may also affect RBC assays. Further studies will also have to relate VFs and MFs to these factors.

This variability obviously affects the ability to detect differences in VFs or MFs between groups in epidemiological studies. A systematic analysis of required sample sizes, given various coefficients of variation, has been accomplished for the *HPRT* cloning assay (Robinson *et al.*, 1994). Similar analyses must be undertaken for all of the systems.

Superimposed on the biological variability is the technical variability. Cryo- or other standards must be developed and validated over time for all of the assays used in epidemiological studies.

Somatic mutations as biomarkers of exposure

Exposure/dose assessment. Somatic mutations in reporter genes are used to assess mutagen/carcinogen exposures. A major determinant of their utility in this regard is their sensitivity relative to other biological end-points. Although mutations do detect exposures, it has been shown that, compared to other measures, e.g. metabolites or protein or DNA adducts, they are among the least sensitive for chemical mutagens/carcinogens (Tates *et al.*, 1991). This might have been expected as the metabolites and adducts are more proximal to the exposures. However, even for acute ionizing irradiation, the most sensitive of the somatic mutation assays is no

better and probably less sensitive than are chromosome aberrations. The reason is that the latter, although clearly reflecting a genotoxic effect, is a very large target, i.e. the entire genome.

Although lacking in sensitivity, there are occasions when reporter gene mutations may be used as exposure dosimeters. First, those assays that are simple and require only small blood samples may be easier and less expensive to use than other biomarkers. Moreover, mutations may be used for exposure assessment for unknown mutagens/carcinogens when no other biological end-points are available. Another use for certain somatic mutations for exposure/dose assessment may be for dose reconstructions of remote exposures, as in nested case–control studies. However, only mutation assays that detect events in stem cells, i.e. the RBC assays, will be useful in this regard. Finally, somatic mutations in the fetus, i.e. as *HPRT* in T-cells, may be useful for population exposure assessments because the newly induced mutations arise on a low and characteristic background.

Exposure characterization. Somatic mutations as biomarkers of exposure may have their greatest utility in terms of exposure characterizations. Mutation spectra are being defined with the expectation that the naturally occurring background spectrum will differ from those produced by different mutagens or classes of mutagens. If so, characterizing the mutations will allow a diagnosis of a specific exposure, i.e. will provide specificity. This will be possible only for those mutations that are recovered for molecular analyses—currently requiring the T-cell assays and laborious tissue culture methods.

It has been shown that ionizing radiation produces a characteristic spectrum of deletions for both *HPRT* and *HLA* mutations. A good deal of effort is now being expended in defining chemical molecular mutational spectra using both in-vivo and in-vitro systems. The identification of such spectra for exposure characterizations may become an important reason for mutagenicity monitoring in humans.

Somatic mutations for detecting genotoxic effects

Somatic mutations unequivocally reveal genotoxic effects. Although such effects in reporter genes do not necessarily indicate genotoxic effects in cancer genes, the most important potential application of reporter gene mutations for human biomonitoring may eventually be as surrogates for cancer mutations. For this to become a reality, it must be demonstrated that mutations in reporter genes, measured in tissues of convenience, are valid surrogates for mutations in cancer genes occurring in target tissues.

Current evidence that reporter gene mutations are valid surrogates in this regard is indirect but positive. First, there is the analogy to another biomarker of effect, namely non-specific chromosome aberrations. Two recent retrospective follow-up studies have shown that individuals with high frequencies of non-specific chromosome changes have relative risks of developing cancer in the next decade of greater than 2.0 (Hagmar *et al.*, 1994; Bonassi *et al.*, 1995). Regression analyses indicated that the aberrations conferred risk beyond that associated with exposure *per se* (Bonassi *et al.*, 1995). It is noteworthy that SCE frequencies, which often detect exposures more sensitively, were not associated with increases in cancer risk.

Animal studies have shown that agents that produce cancers in various tissues also produce *HPRT* T-cell mutations *in vivo* (Aidoo *et al.*, 1991). Thus, *HPRT* is a functional surrogate for cancer in these species. Finally, the administration of radioprotective agents in mice receiving ionizing radiation reduced both the induced malignancies and *HPRT* T-cell mutations, again relating the reporter events to disease-causing events (Grdina *et al.*, 1991, 1992).

The best evidence that reporter mutations reflect the occurrence of cancer gene mutations in humans is the discovery that mutagenic mechanisms with carcinogenic potential are captured in these reporter genes. The occurrences of the somatic recombinations, the deletions with characteristic breakpoints, the fusion genes and the V(D)J recombinase-mediated mutations in the various reporter genes have been described.

The use of the various assays detecting events in cancer genes for epidemiological studies remains to be defined.

Future directions

Next-generation assay development

The next generation of assay development will probably involve both reporter and cancer genes. Reporter genes are useful for defining the molecular

ases of in-vivo geneti
ssays that allow this are
orm. New developme
ethods to identify and
o not require tissue cult
The five current repor
se only blood cells. The d
ased assays will also all
on-selectable genes and
f other tissues. Eventua
um can be developed,
method to design and cha
olecular technologies
cale human studies.

The development of mu
n cancer genes is just now
evelopment will likely fo
utations or mutated pr
nd sensitive immunologi
require further methodol

Increased understanding of somatic mutation

The interpretation of soma
populations will be enhan
standing of the biological
nd of how this relates to
Advances in our understa
asis for several of the maj
dromes have led to an app
ying mechanisms for the
somatic mutations in the
echanistic links may be
bove for V(D)J recombina
demonstrated that transfec
vitro with HPV proteins m
ncrease mutagenesis of th
(Havre *et al.*, 1995). Thus,
human populations may be
tions as well as by chemic
exposures and underlying g
Studies in human populatio
anisms for somatic cell muta
an understanding of these in
consequently of a possible r

Although the 'memory'
appears to diminish with ti
quence of development, app
eral blood (or other potenti
and eventual disappearance

bases of in-vivo genetic damage. However, the assays that allow this are costly and difficult to perform. New developments will therefore be in methods to identify and quantify mutations that do not require tissue culture.

The five current reporter gene mutation assays use only blood cells. The development of molecular-based assays will also allow the sampling of other non-selectable genes and will include examination of other tissues. Eventually, a large armamentarium can be developed, using the tissue culture method to design and characterize systems and the molecular technologies for application to large-scale human studies.

The development of mutation assays for changes in cancer genes is just now beginning. Future assay development will likely focus on exposure-specific mutations or mutated protein products. Specific and sensitive immunological and molecular assays require further methodological development.

Increased understanding of the biological process of somatic mutation

The interpretation of somatic mutations in human populations will be enhanced by increased understanding of the biological basis of their occurrence and of how this relates to human carcinogenesis. Advances in our understanding of the biological basis for several of the major gene instability syndromes have led to an appreciation of the underlying mechanisms for the frequent occurrence of somatic mutations in these syndromes. Other mechanistic links may be expected, as indicated above for V(D)J recombinase. It has recently been demonstrated that transfection of human cells *in vitro* with HPV proteins may inactivate *p53* and increase mutagenesis of the *HPRT* reporter gene (Havre *et al.*, 1995). Thus, somatic mutations in human populations may be determined by infections as well as by chemical/ physical mutagen exposures and underlying genetic susceptibilities. Studies in human populations exploring the mechanisms for somatic cell mutation will contribute to an understanding of these interrelated factors, and consequently of a possible relation to cancer.

Although the 'memory' of somatic mutations appears to diminish with time, the temporal sequence of development, appearance in the peripheral blood (or other potentially assessed tissues), and eventual disappearance of somatic mutations are poorly understood. As our understanding of cell replication and death increases, it may be possible to distinguish more clearly long-term mutational events. This would clearly be advantageous for the purposes of dosimetry. This insight, however, may also be relevant for understanding the time-dependent relationship between exposure and the development of cancer. For example, radiation-induced leukemia is detectable after just a few years, reaches a peak at about 5 years after exposure, and decreases thereafter (Committee on the Biological Effects of Ionizing Radiation, 1990). A similar pattern, but with a substantially longer wave period, has been described for radon-induced lung cancer (Lubin *et al.*, 1994). Studies to evaluate the time-dependent occurrence of somatic mutations in cell populations may provide insight into this process.

With the further development of molecular approaches to somatic mutation assessment, it will increasingly be possible to investigate multiple markers in human studies. This approach can provide internal consistency checks and may broaden mechanistic understanding.

Validation of somatic mutations as predictors of disease outcome

A limitation for the interpretation of the reporter gene somatic cell mutation assays is uncertainty about the significance of these events as surrogates for mutations leading to cancer in humans. For mutations in cancer genes, problems in interpretation also arise because the mutations observed may not lead to functional alterations or may be 'after the fact', i.e. may indicate existing but subclinical cancer, and therefore not be useful for the purposes of prevention.

With improvements in assay methodology, it will increasingly be possible in large-scale epidemiological studies to determine if specific somatic cell mutations are statistically associated with increased risks for cancer. Case–control studies can be used for this purpose, but will probably be of limited value for the 'short memory' markers and because of the possibility that disease status may influence marker outcome. 'Nested' case–control studies of a subset of cases and comparison subjects for whom samples were collected prior to disease development as part of a large cohort investigation provide a methodologically sound alternative to

the case–control study. Several such cohorts have now been established, but procedures for the collection and storage of biological materials are suitable only for molecular studies and not for the standard cell-based assays, unless special procedures such as cryopreservation are undertaken. Although a case can be made for the special storage procedures, they are laborious and expensive. Because of this, direct validation studies will usually require molecular approaches.

While such validation studies are clearly needed to describe the statistical relationship between somatic mutations and cancer risk, a positive association does not establish a causal link. Ultimately, the usefulness of the somatic cell mutation assays will be based upon our understanding of their biological basis and of how this relates to human carcinogenesis.

References

Aidoo, A., Lyn-Cook, L.E., Mittelsyaedt, R.A., Heflich, R.H. & Casciano, D.A. (1991) Induction of 6-thioguanine-resistant lymphocytes in Fischer 344 rats following *in vivo* exposure to *N*-ethyl-*N*-nitrosourea and cyclophosphamide. *Environ. Mol. Mutagen.*, 17, 141–151

Akiyama, M., Kyoizumi, S., Hirai, Y., Hakoda, M., Nakamura, N. & Awa, A.A. (1990) Studies on chromosome aberrations and HPRT mutations in lymphocytes and GPA mutation in erythrocytes of atomic bomb survivors. In: Mendelson, M.L. & Albertini, R.J., eds, *Mutation and the Environment Part c: Somatic and Heritable Mutation, Adduction, and Epidemiology, Progress in Clinical and Biological Research, 340c*, New York, Wiley-Liss, pp. 69–80

Albertini, R.J. (1985a) Somatic gene mutations *in vivo* as indicated by the 6-thioguanine-resistant T-lymphocytes in human blood. *Mut. Res.*, 150, 411–422

Albertini, R.J. (1985b) The use of human T-lymphocytes to monitor mutations in human populations. In: Li, A.P., ed., *New Approaches in Toxicity Testing and Their Application in Human Risk Assessments*. New York, Raven Press. pp. 51–66

Albertini, R.J., Allen, E.F., Quinn, A.S. & Albertini, M.R. (1981) Human somatic cell mutation: *in vivo* variant lymphocyte frequencies determined by 6-thioguanine resistant lymphocytes. In: Hook, E.B. & Potter, J.H., eds, *Population and Biological Aspects of Human Mutation*, New York, Academic Press, pp. 235–263

Albertini, R.J., Castle, K.L. & Borcherding, W.R. (1982) T-cell cloning to detect the mutant 6-thioguanine-resistant lymphocytes present in human peripheral blood. *Proc. Natl Acad. Sci. USA*, 79, 6617–6621

Albertini, R.J., Sullivan, L.S., Berman, J.K., Greene, C.J., Stewart, J.A., Silveira, J.M. & O.'Neill, J.P. (1988) Mutagenicity monitoring in humans by autoradiographic assay for mutant T-lymphocytes. *Mut. Res.*, 204, 481–492

Albertini, R.J., Nicklas, J.A., O.'Neill, J.P. & Robison, S.H. (1990) *In vivo* somatic mutations in humans: measurement and analysis. *Ann. Rev. Genet.*, 24, 305–326

Ammenheuser, M.M., Ward J.B., Jr, Whorton, E.B., Jr, Killian, J.M. & Legator, M.S. (1988) Elevated frequencies of 6-thioguanine resistant lymphocytes in multiple sclerosis patients treated with cyclophosphamide: a prospective study. *Mut. Res.*, 204, 509–520

Ammenheuser, M.M., Au, W.W., Whorton E.B., Jr, Belli, J.A. & Ward J.B., Jr (1991) Comparison of *HPRT* variant frequencies and chromosome aberration frequencies in lymphocytes from radiotherapy and chemotherapy patients: a prospective study. *Environ. Mol. Mutagen.*, 18, 126–135

Anderson, D., Hughes, J.A., Brinkworth, M.H., Peltonen, K. & Sorsa, M. (1996) Levels of *ras* oncoproteins in human plasma from 1,3-butadiene-exposed workers and controls. *Mut. Res.*, 349, 115–120

Bachand, M., Seifert, A.M. & Messing, K. (1991) Nuclear medicine patients do not have higher mutant frequencies after exposure thallium-201. *Mut. Res.*, 262, 1–6

Bernini, L.F., Natarajan, A.T., Schreuder-Rotteveel, A.H.M., Giordano, P.C., Ploem, J.S. & Tates, A. (1990) Assay for somatic mutation of human hemoglobins. In: Mendelson, M.L. & Albertini, R.J., eds, *Mutation and the Environment Part c: Somatic and Heritable Mutation, Adduction, and Epidemiology, Vol Progress in Clinical and Biological Research, 340c*, New York, Wiley-Liss, pp. 57–68

Bigbee, W.L., Branscombe, E.W., Weintraub, H.B., Papayannapoulou, T. & Stamatoyannopoulos, G. (1981) Cell sorter immunofluorescence detection of human erythrocytes labeled in suspension with antibodies specific for hemoglobin S and C. *J. Immunol. Methods*, 45, 117–127

Bigbee, W.L., Wryobek, R.G., Langlois, R.G., Jensen, R.H. & Everson, R.B. (1990) The effect of chemotherapy on the in vivo frequency of glycophorin A "null" variant erythrocytes. *Mut. Res.*, 240, 165–175

Bigbee, W.L., Grant, S.G., Langlois, R.G., Jensen, R.H., Anttila, A., Pfaffli, P., Pekari, K. & Norppa, H. (1996) Glycophorin A (*GPA*) somatic cell mutation frequencies in Finnish reinforced plastics workers exposed to styrene. *Cancer Epidemiol. Biomarkers Prev.*, 5, 801–810

Bodmer, W.F. (1984) The HLA system, 1984. In: Albert, E.D., Baur, M.P. & Mayr, W.R., eds, *Histocompatibility Testing 1984*, Berlin, Springer Verlag, pp. 11–22

Boehm, T. & Rabbitts, T.H. (1989) The human T cell receptor genes are targets for chromosomal abnormali-