9 *In Vivo* Somatic Cell Gene Mutations in Humans

R. J. Albertini, J. A. Nicklas, S. H. Rebison, and J. P. O'Neill*
University of Vermont, Burlington, Vermont, USA

9.1 INTRODUCTION

9.1.1 RATIONALE

In humans, gene mutations in somatic cells probably underlie a variety of developmental and pathological processes. Among the latter are cancer and, possibly, aging. Disease relevance alone provides motivation for undertaking studies to understand mechanisms by which mutations arise, and is a powerful incentive to conduct studies of their *in vivo* occurrence in humans.

In addition, assays for *in vivo* mutations in indicator genes are useful tools in genetic toxicology and risk assessment. Such assays may allow quantitative and qualitative assessments of adverse effects in human populations, and may even enable identification of the nature of toxic exposures. *In vivo* mutagenicity studies may identify heterogeneity of susceptibility among humans for specific or classes of mutagens or carcinogens. Ultimately, relating *in vivo* mutations in indicator genes to occurrences of subsequent genotoxic diseases such as cancer or birth defects may be possible. As this becomes possible, estimates of relative risk may be made for individuals exposed to deleterious environmental agents, providing a basis for medical intervention such as removal from the source, early diagnosis, or, in the case of cancer, application of chemopreventive measures. Somatic mutations arising *in vivo* in humans are, therefore, worthy of study for their biological as well as toxicological significance.

9.1.2 HISTORICAL CONTEXT

* The research reported herein was supported by US NCI ROI CA 30688 and US DOE FG02 87 60502; this support does not constitute an endorsement by DOE of the views expressed in this paper.

Methods to Assess DNA Damage and Repair: Interspecies Comparisons
Edited by R. G. Tardiff, P. H. M. Lohman and G. N. Wogan

The mouse "spot-test" was the first useful assay to quantify *in vivo* somatic cell gene mutations in mammals (Russell and Major, 1957). Only a year later, a less well-known report described an *in vivo* assay in humans that detected loss of ABO antigens from red blood cells (RBC; Atwood and Scheinberg, 1958). These variant cells were considered to be somatic cell mutants. Two additional types of variant cells containing altered hemoglobin, found at low frequencies in human peripheral blood, were reported over the next 15 years (Sutton, 1972, 1974; Stamatoyannopulos *et al.*, 1975; Wood *et al.*, 1975). Although these assays for variant cells arising *in vivo* were initially proposed as measures of somatic mutations in humans, all were eventually rejected for this purpose, because the cellular changes could never be demonstrated to have been the result of gene mutations. Similar variants could result from non-genetic causes (Atwood and Petter, 1961; Papayannopouou *et al.*, 1977a, 1977b), i.e., each assay was contaminated with "phenocopies" (cells which exhibit a mutant phenotype in the absence of the mutant genotype).

9.1.3 CURRENT ERA

Four assays to detect *in vivo* gene mutations in human somatic cells now exist, and each addresses phenocopies. Results of studies using these assays indicated that they actually defined mutant cells; however, the assays enumerate mutant cells, not the underlying mutational events. For most toxicological evaluations, the mutations themselves are most important. At best, frequencies of mutant cells correlate only imperfectly with frequencies of mutations, because the latter may yield one or more mutants per event, depending on cell division. This effect is compounded when spontaneous mutations may occur preferably in dividing rather than quiescent cells. This issue is considered in detail when discussing each assay.

9.2 CURRENT ASSAYS FOR HUMAN *IN VIVO* SOMATIC CELL MUTATIONS

All four assays currently used for *in vivo* studies of human somatic cell gene mutations measure changes in peripheral blood cells, either RBCs or lymphocytes. Although blood is a useful tissue for human mutagenicity monitoring because of the ease of sample acquisition, exclusive reliance on blood cells for measuring *in vivo* mutation results in serious deficiencies. Most human malignancies, for instance, do not arise in blood cells; the common human cancers are of epithelial origin. For toxicity studies, therefore, information obtained from blood cells must be extrapolated to other tissues; yet no information exists regarding the validity of such extrapolations. Thus, research in this area is of paramount importance.

9.2.1 RBC ASSAYS

RBCs are abundant in human blood, and are easily analyzed for the presence of rare variants. However, since a mature RBC has lost its nucleus and, hence, its DNA, any gene mutation seen in an RBC must have arisen *in vivo* in precursor cells. Of necessity, this implies some distance (in terms of cell divisions) between mutational events and measured variants. Furthermore, the possibility for molecular analyses of variants is eliminated. The RBC assays rely on indirect means to avoid phenocopies and to relate mutant frequencies to underlying mutational events.

9.2.2 HEMOGLOBIN MUTANTS

Of the current human *in vivo* mutation assays, the detection of altered haemoglobin in RBCs was the first to be proposed (Stamatoyannopoulos *et al.*, 1984; Bigbee *et al.*, 1983, 1984). Hemoglobin, a tetrameric protein consisting of two heterodimers, is encoded by genes at two sets of linked loci on chromosomes 11 and 16 (Deisseroth *et al.*, 1977, 1978). Most normal post-natal human haemoglobin is designated haemoglobin A (HBA, α_2; β_2) with a small amount of haemoglobin A_2 (α_2; δ_2).

Mutations of altered haemoglobin polypeptide chains produce specific and unambiguous changes. Such changes cannot be produced as phenocopies. More than 300 such mutations have been defined at the germinal level (Weatherall, 1985), each of which can serve in principle as a marker for *in vivo* somatic cell gene mutation. The best known example is mutation of the β gene (chromosome 11) that produces sickle-cell haemoglobin (HbS). This mutation is an A→T base change that causes the amino acid valine to be substituted for glutamine at position 6 of the β polypeptide. Therefore, although the β gene contains three exons and spans 2 kb, the effective target size for the HbS mutation is a single base pair.

Quantitatively relating RBC haemoglobin variants to underlying somatic mutational events is complex (Stamatoyannopoulos *et al.*, 1984). This degree of complexity holds for all mutations detected in mature RBCs. If the *in vivo* mutations responsible for variant RBCs arise in pluripotent or multipotent stem cells, a factor determining the frequency of variant cells at any time is the number of stem cells generating the RBC population at that time. If this number is small, the many cell divisions between the mutations and the sampled RBCs may produce large interindividual variations in variant frequency (Vf) values. Likewise, for mutations in pluripotent stem cells, large interindividual variations of RBC Vf may exist following identical exposures. Such variability is due to the cell stage at which mutations occur, and will be reduced if somatic mutations occur early in the development of the individual (i.e., before even the entire stem cell complement is generated), and occurs repeatedly as a result of chronic exposure. The advantage to stem cell mutations is memory, i.e., mutant progeny are produced continuously.

On the other hand, if the mutations reflected in RBCs arise *in vivo* in committed RBC precursors (rather than in true stem cells), the relationship between mutations and mutants will be more direct. However, such mutations will produce mutants

only transiently, because these precursor cells themselves are lost with time. The disadvantage of mutation in differentiated cells is loss of memory, while the advantages are close temporal relationships of Vf elevations to mutagen exposures and less variability in Vf values.

Stamatoyannopoulos and colleagues (1984) originally developed specific antibodies to detect HbS-containing RBCs. Their early methods depended on manual reading of slides to detect rare variant RBCs. Recently, a method that treats fixed RBCs on slides with polyclonal anti-HbS antibodies and uses automated image analysis to screen large numbers of cells to detect rare RBCs labelled with anti-HbS fluorescent antibodies has been reported (Verwoerd *et al.*, 1987). Using this technique, Tates and coworkers (Tates *et al.*, 1989) have measured HbS Vf for several groups of individuals: normal non-smoking adults, smokers, xeroderma pigmentosum (XP) and ataxia telangectasia (AT) patients and heterozygotes, and workers exposed to ethylene oxide.

These preliminary results establish the background of HbA → HbS Vf to be approximately 5×10^{-8} for normal adults. Such a low frequency, although expected for the change of only a single base, creates the technical difficulty that extremely large numbers of cells must be analyzed to obtain interpretable data. This difficulty may be mitigated by simultaneous assays for several different mutant hemoglobins using a mixture of several different antibodies (Tates *et al.*, 1989).

9.2.3 GLYCOPHORIN A (GPA) LOSS MUTANTS

GPA is a glycosylated cell surface protein (approximately 10^5 molecules per RBC) that carries the M and N blood group antigens (Furthmayer, 1977; Gahmberg *et al.*, 1979). M and N, which differ by two non-adjacent amino acids, are concomitantly expressed and represent the only two alleles of the GPA locus. The gene for GPA is located on human chromosome 4, has seven exons, and spans more than 44 kb (Kudo and Fukuda, 1989).

Highly specific anti-M and anti-N antibodies linked with green and red fluorophors, respectively, label the wild type RBCs from M/N heterozygous individuals with double fluorescence. Scoring with a fluorescence activated cell sorter permits rapid enumeration of rare RBCs that have lost either antigen. A GPA loss Vf is defined as the number of single colour cells (green or red) divided by the total number of RBCs scored (single plus double fluorescence). In principle, this assay measures large targets, detecting a variety of mutations that inactivate the GPA gene. However, the target size cannot be known precisely because of the protein structure required for antibody binding.

In addition to simple M or N losses, another class of GPA variants found in heterozygous individuals is characterized by the loss of expression of one allele (M or N), with double expression of the other. Such variants, called "homozygous variants" (as opposed to simple loss, or "hemizygous variants") are interpreted as products of somatic crossing-over or gene conversion.

The GPA assay has internal safeguards against the scoring of phenocopies. For a cell to be recorded, one antigen (M or N) must be detected in RBCs from an M/N individual. Non-genetic, or genetic non-GPA, locus events should result in loss of all GPA proteins. Although only M/N heterozygotes can be studied in the GPA assay, the M and N alleles have approximately equal gene frequencies in all populations. Therefore, 50% of individuals are suitable for study.

Most results with the GPA assay are obtained from the Lawrence Livermore National Laboratory, and are based on use of a dual beam flow cytometer (Jensen *et al.*, 1987; Langlois *et al.*, 1989b). GPA results are usually expressed as either hemizygous variants (gene loss) or homozygous variants (recombinants). Mean Vf values for normal non-smoking adults are approximately 10×10^{-6}, with rather wide interindividual (but narrow intraindividual) variation. Mean Vf values in smokers are approximately 30% above those of non-smokers; however, this difference is significant only to the 0.1% statistical level. Hemizygous and homozygous variants occur with equal frequencies in normals. An age effect occurs for GPA Vf values resulting in a linear rise in Vf values of approximately 2% per year, with Vf values at birth (i.e., measured in placental cord blood) falling on the calculated regression line. *In vivo* GPA Vf values have been reported for several DNA-repair-defect syndromes (Langlois *et al.*, 1989a, 1990; Bigbee *et al.*, 1989; Kyoizumi *et al.*, 1989a).

The results with XP patients and heterozygotes may be explained by the site of GPA mutations, i.e., in RBC precursors in bone marrow. Probably little opportunity exists for UV-light to penetrate this body compartment. Alternatively, these results may reflect specific characteristics of the target GPA gene. The 10-fold increase in Vf seen in AT homozygotes and the 100-fold increase seen in Bloom's syndrome (BS) patients parallels their known propensity to show chromosome breakage and translocations. The increase in numbers of homozygous variants in BS patients is believed to be due to *in vivo* somatic gene recombination events reflecting the cancer predisposition of BS patients. None of the heterozygotes for these repair-deficiency syndromes showed elevated Vf values.

Mean GPA Vf values for cancer patients prior to chemo- or radiotherapy showed no differences over comparable controls (Bigbee *et al.*, 1990a). However, longitudinal studies in breast cancer patients receiving adjuvant chemotherapy regimens showed Vf increases after an initial latency of 1 to 3 weeks. At most, these increases approached eight-fold over background, and returned to baseline 120 days after cessation of therapy. This return to baseline presumably indicates that chemically induced GPA variants arise from *in vivo* mutations in committed RBC precursors rather than in pluripotent stem cells.

Several cancer patients receiving a variety of high dose, external beam, localized radiotherapy regimens were studied by GPA assay, and no elevations in Vf have been observed (Mendelsohn, 1991). Perhaps such localized bone marrow radiation produces massive lulling of stem cells, with few survivors for production of mutants. These findings differ from those of atomic bomb survivors studied by GPA assay more than 40 years after exposure; on average, they showed dose-

dependent increases in GPA Vf (approximately 40 variants/10^6 RBCs per Gray). This finding was duplicated by another laboratory in Hiroshima (Kyoizumi *et al.*, 1989b) using a single beam cell sorter. The significance of this finding include not only confirmation of the initial results but also demonstration that this simpler, more widely available instrument is suitable for the GPA assay. As expected, in both Hiroshima studies, large interindividual variations were observed, presumably reflecting: (a) the occurrence of mutations in stem cells; (b) the limited numbers of such cells available for mutations; and (c) cell killing in this limited pool.

A preliminary report of GPA mutations in individuals in the Chernobyl area presented 9 months after the accident revealed a dose-dependent (based on dicentric chromosomes) increase in GPA Vf values (Langlois *et al.*, 1990). The shape of the dose–response curve agreed within a factor of 2 with the Hiroshima data (Mendelsohn, 1991). Wide scatter existed in the data, with the highest Vf being 536×10^{-6}. Most of the increase in variants in Chernobyl individuals was of the hemizygous rather than the homozygous type, suggesting deletions rather than crossing-over events.

These results demonstrated that the GPA assay is operational, should soon be available to study more groups, and will continue to provide information regarding *in vivo* somatic gene mutation in human RBC precursors. The utility of the RBC assays rests on the speed and ease with which many cells may be scored. The lack of genetic material precludes further molecular analyses; recent advances in the use of reticulocytes in this assay may allow molecular analysis of some of the mutations (DuPont *et al.*, 1991; Langlois *et al.*, 1991).

9.2.4 LYMPHOCYTE ASSAYS

T lymphocytes are nucleated cells, also readily available in blood. T lymphocytes can be easily expanded *in vitro* by the use of T-cell growth factors and feeder cells. DNA can be extracted from these cells for molecular studies, including determination of mutational spectra.

9.2.4.1 *hpri* Mutants

Rare 6-thioguanine-resistant (TG) T lymphocytes in peripheral blood arise *in vivo* by mutation at the gene for hypoxanthine-guanine phosphoribosyltransferase (HPRT; *hprt).* HPRT mediates the phosphoribosylation of hypoxanthine and guanine for reutilization of purines. This enzyme is constitutive but non-essential. HPRT can also phosphoribosylate purine analogues such as TG or 8-azaguanine, causing these analogs to become cytotoxic. Thus, loss of HPRT activity can be selected by resistance to such analogs.

The *hprt* gene is located on the X-chromosome (Xq26) (Henderson *et al.*, 1969), and is approximately 44 kb in size and contains nine exons (Patel *et al.*, 1986).

The entire gene region has been sequenced (Edwards *et al.*, 1990). A large background of information exists on *hprt* mutations *in vitro* in human and other mammalian cell systems (Wilson *et al.*, 1986). The *hprt* T-cell assays were the first to become operational for human *in vivo* mutation studies; consequently, much quantitative and molecular information supports the use of this system.

Short-term DNA replication assays

Hprt mutant T cells arising *in vivo* can be detected by two short-term assays. One method uses autoradiography to detect ^{3}H-thymidine incorporation in mutant T cells that are able to overcome TG inhibition of first-round phytohemagglutinin (PHA) stimulated DNA synthesis *in vitro* (Strauss and Albertini, 1979). The original autoradiographic assay allowed pseudoresistant T cells to be scored as mutants (Albertini *et al.*, 1981; Albertini, 1985). These phenocopies appeared to arise because TG cannot totally block the first round of *in vitro* DNA synthesis in actively cycling T lymphocytes, as contrasted to its effect in resting cells. Several ways exist to eliminate this problem (Albertini *et al.*, 1981; Albertini, 1985; Amneus *et al.*, 1982, 1984; Matsson *et al.*, 1985; Zetterberg *et al.*, 1982). All depend to some extent on elimination of *in vivo* dividing T cells from enumeration in the assay. Because of its simplicity, the autoradiographic assay is useful for population screening, is relatively inexpensive, and has the potential for automation (Stark *et al.*, 1984; Amneus *et al.*, 1982, 1984; Matsson *et al.*, 1985; Zetterberg *et al.*, 1982). Equally important, a recently developed short-term method has been developed; it incorporates BrUdR followed either by Hoechst staining (dye #33258) or detection by antibodies to score for TG cells, thereby eliminating the need for autoradiography.

Using autoradiographically determined *hprt*, background Vf values for normal adults have now been reported by several groups using cryopreservation to eliminate the scoring of phenocopies. Ostrosky-Wegman *et al.* (1987) found a mean Vf value of 6.9×10^{-6} for 18 normal adults; Ammenheuser *et al.* (1988) reported a mean *hprt* Vf of 1.9×10^{-6} for eight normal adults; and Albertini (1985) gave 8.7×10^{-6} as a mean value for 82 assays of normal individuals. A sub-group of 26 individuals within this last cohort demonstrated an age effect, with an increase in Vf of 0.26×10^{-6} per year (or approximately 5% per year where the Vf value is 5×10^{-6}).

Amneus and coworkers (Amneus *et al.*, 1982; Amneus and Erikson, 1986) used a cell sorter to enrich for labelled TG T cells in the autoradiographic assay. The window used in sorting cells after short-term culture in TG included nuclei with twice the DNA content. This served to eliminate phenocopies, which usually are able to progress only to early S-phase in the presence of TG. These workers reported a background Vf of 3 to 5×10^{-5} for non-mutagen exposed adults, a value three to 10-fold different from that reported using cryopreservation and slide scoring. The reason for this discrepancy is unknown.

Chemotherapy and radiotherapy in cancer patients have usually resulted in elevated autoradiographically determined *hprt* T-cell Vf values. In multiple sclerosis patients, Ammenheuser *et al.* (1988) reported 14 days after cyclophosphamide treatments an increase in mean Vf from 4.1×10^{-6} to a range of values between 11.6 and 40.3×10^{-6}. However, over 2 to 4 months, these elevated values fell to baseline levels. These declines are consistent with the induction of mutations primarily in mature, differentiated T lymphocytes, and is interpreted as evidence for *in vivo* selection against *hprt* mutant cells. Whether this phenomenon is generalized or the result of the cytotoxic effects of cyclophosphamide treatment is unknown. These workers also reported elevated Vf values after external beam radiotherapy in cancer patients, rising from a mean of 3.2×10^{-6} (n = 7) before treatment to 17.7×10^{-6} four weeks after treatment (Ammenheuser *et al.*, 1989, 1991). Again, increases in Vf value were not seen until 14 days post-treatment. Ostrosky-Wegman *et al.* (1987) reported elevated Vf values in three persons 1 year after exposure to ^{60}Co-external-beam γ-irradiation.

Cloning assay

A disadvantage of the autoradiography assay is that mutant cells cannot be recovered; therefore, the direct cloning assay has been developed to study mutations in T lymphocytes (Albertini *et al.*, 1982; Morley *et al.*, 1983, 1985; Messing and Bradley, 1985; O'Neill *et al.*, 1987; Cole *et al.*, 1988; Hakoda *et al.*, 1988). T lymphocytes are cultured in limiting dilutions in the absence and presence of TG in wells of microlitre plates. To culture medium and TG, wells contain a source of growth factor (interleukin-2; IL-2) and X-irradiated feeder cells. Cloning efficiencies are determined from the Poisson relationship $P_0 = e^{-x}$ where P_0 is the proportion of wells without growing colonies and "x" is the calculated average number of clonable cells per well. The calculated mutant frequency (Mf) is the ratio of the cloning efficiency in the presence to the cloning efficiency in the absence of TG. Growing colonies can be isolated and propagated *in vitro* to sufficient numbers for molecular and other analyses.

Several laboratories have studied background *hprt* Mf in normal subjects using the clonal assay. The results are consistent, with mean values for adults ranging from 3 to 10.1×10^{-6} (mean = 5.3 ($\pm$ 2.7)$\times10^{-6}$. Clearly, an age-related increase in Mf was seen, and was most pronounced for newborns for which background mean *hprt* Mf values were considerably less than expected from simple regression. After the newborn period, Mf values increase 1.7 to 5%.

Chemotherapy in cancer patients also caused significant increases in *hprt* Mf values. Eleven patients receiving chemotherapy alone had elevated Mf compared with 42 healthy controls (19.57 versus 6.72×10^{-6}, respectively), while combination chemotherapy–radiotherapy resulted in a further mean Mf elevation to 34.4×10^{-6} (Dempsey *et al.*, 1985). These mutagenic effects of combined therapy appear to result primarily from the radiotherapy component (Sala-Trepat *et al.*, 1990).

Ionizing radiation also elevated *in vivo* Mf values (Sanderson *et al.*, 1984; Messing *et al.*, 1986, 1989; Seifert *et al.*, 1987). A study of therapeutic external beam, ionizing radiation showed *hprt* mutants to be produced at a rate of 23×10^{-6}/Gray (Sanderson *et al.*, 1984). Patients receiving chronic, total body radioimmunotherapy (RIT) with ^{131}I, ^{90}Y, or a combination of the two as internal emitters also showed elevated *hprt* Mf values (Nicklas *et al.*, 1990). Patients who received ^{90}Y showed Mf values ranging from normal to 42×10^{-6}, while those who received ^{131}I showed elevated Mf values in all cases with a mean for 21 patients of 90.2×10^{-6} (an eight- to 10-fold elevation over normal young adults). The RIT study showed good correlation between Mf values and initial doses of administered radioactivity. However, this correlation of Mf to total exposures was poor after several rounds of treatment. A blind study of the *hprt* mutant frequency from relatively low atmospheric concentrations of ionizing irradiation in the form of household radon exposure has been undertaken recently. Such studies are relevant for human risk assessment, and should be undertaken in a wide range of populations exposed to radon.

A study of atomic bomb survivors conducted 45 years after the exposure showed a significantly elevated *hprt* Mf value in exposed individuals (Hakoda *et al.*, 1989a). Although a dose–response relationship was present between Mf and exposure level, the slope was very shallow (2 to 3 mutants/10^6 cells per Gray) as compared with the responses observed in the GPA assay. This observation most likely reflects the different sites for mutations in RBCs and T lymphocytes with a subsequent loss of *hprt* but not GPA mutants.

Most quantitative studies of induced *in vivo hprt* mutations appear to show a decline in mutant cells with time after treatment. This effect could be due to either of the following: (a) negative *in vivo* selection against mutant cells, possibly as a consequence of cytotoxic treatments resulting in lymphocyte proliferation; or (b) a characteristic of mutations in committed, mature cells. The practical consequence of this observation is that elevated *hprt* T-cell mutant frequencies suggest a recent (i.e., less than 6 months) rather than a distant genotoxic exposure.

Several laboratories are investigating *in vivo hprt* mutations at the DNA level, and a repository for these molecular data is being established (Albertini *et al.*, 1989; Craft *et al.*, 1991). Several reports of analyses at the Southern blot level now exist, with surprising agreement that large alterations account for no more than 15% of background (i.e., spontaneous) *hprt* mutants recovered from adults. Most gross *hprt* alterations are deletions, with some being more numerous than total deletions.

Analyses of gross structural alterations allow inferences regarding the nature of spontaneous *in vivo* deletions. One inference concerns the maximum size deletion that can be tolerated at *hprt*. Twenty-eight simple deletions analyzed by Nicklas *et al.* (1989) showed that the intragenic breakpoints were evenly distributed across the gene. This finding, coupled with the finding that approximately 50% of the breakpoints occurred outside of the gene, suggested that spontaneous *hprt* deletions of at least 94 kb could be recovered. Additional X-linked DNA probes have subsequently been used to define the extent of viable multi locus deletions that may

accompany *in vivo hprt* mutations in T cells. Thus far, losses of linked markers that may map up to 10 cm (~10 Mb) distant from *hprt* by linkage analysis have been detected (Nicklas *et al.*, 1991). This observation requires re-examination of the hypothesis that *hprt* is insensitive to mutational events resulting from large deletions.

Mutations (*hprt*) have been isolated from the human fetus via placental cord blood. McGinniss *et al.* (1989) reported that gross structural alterations were detected in 85% of placental mutant isolates, with most deletion mutations involving loss of *hprt* exons 2 and 3, a finding confirmed in a larger study (Lippert *et al.*, 1990). The spontaneous *hprt* mutations in the fetus have a characteristic molecular spectrum even at this gross level of analysis. These unique intragenic deletions of exons 2 and 3 have been studied further by polymerase chain reaction (PCR) of genomic DNA and sequence analysis. In the 13 mutants analyzed, the deletion breakpoints occurred in the same sequences in introns 1 and 3 of the gene. These sequences contained a consensus heptamer which directs DNA cleavage by the V(D)J recombinase, which directs the T-cell receptor (TCR) and immunoglobulin gene rearrangements. In these mutants, during T-lymphocyte differentiation in the fetus, the recombinase has created this illegitimate deletion of exons 2 and 3 in the *hprt* gene—the first example of developmental mutagenesis in a constitutive gene *in vivo* in any species (Fuscoe *et al.*, 1991a, 1991b).

In adults, however, most background T-cell *hprt* mutants arise from point mutations. The PCR procedure is being used to amplify mutant *hprt* cDNA derived from the mRNA of these point mutation isolates, followed by direct sequencing of the products (Recio *et al.*, 1990; Rossi *et al.*, 1990). Observed changes include base-pair changes (transitions and transversions), multiple base gains and losses, frameshift mutations, and exon losses that probably represent splice-site mutations (Albertini *et al.*, 1990). In some instances, the base change(s) responsible for the splice-site mutations have been localized. Data sufficient to define a meaningful background adult *hprt* mutational spectrum are accumulating rapidly.

Molecular studies of *hprt* mutants arising *in vivo* after ionizing radiations are ongoing. All reports involve only analysis at the Southern blot level. Generally, patients receiving chronic therapeutic ionizing irradiations show an increased proportion of their *hprt* mutants with gross structural alterations, and the maximum size of these *hprt* deletions is larger than seen for background mutants (Hakoda *et al.*, 1989a; Nicklas *et al.*, 1990). Mutants isolated from atomic bomb survivors have produced mixed results (Hakoda *et al.*, 1989a). In one survivor, 26% of the mutant isolates showed gross structural changes of *hprt*; in another, the proportion of mutants with gross *hprt* gene alterations was the same as in normals.

The ability to measure *hprt* mutants *in vivo* permits characterization of the spectrum of mutations in humans. The number of mutational events is the appropriate denominator to describe this range. However, genetic changes themselves are characterized in clones of mutants. In cell populations capable of division and when mutations do not inhibit cell division, the frequencies of mutations and mutant progeny are not necessarily identical. Thus, characterization

of multiple mutants arising from the same mutational event is possible. However, in T lymphocytes, TCR gene rearrangement analyses can establish *in vivo* the relationship among *hprt* mutant isolates. T-cell mutants having identical TCR gene rearrangement patterns are related clonally in that they arose *in vivo* in the same clone of cells. Such mutants are referred to as TCR gene defined mutant sets (TCR sets). If mutants in a TCR set also show the identical *hprt* change, then they arose *in vivo* from the same *hprt* mutational event (i.e., they are siblings). All such mutants represent but a single mutation, and should be scored only once when defining a spectrum of mutations.

Molecular analyses of TCR gene rearrangement patterns in *hprt* T-cell mutants are being used to define relationships between mutants and their underlying *hprt* mutations. TCRs are encoded by TCR genes that undergo conformational rearrangements, in a manner similar to immunoglobulin genes, from a germ line to a functionally rearranged pattern during intra-thymic cellular differentiation. An enormous diversity of TCR gene rearrangement patterns are recognizable on Southern blots, with a given pattern marking a differentiated T cell and its clonal descendants.

Pair-wise comparisons from a single individual of different *hprt* mutant isolates (according to both *hprt* change and TCR gene rearrangements) allow estimations of the origin of mutants from multiple independent *in vivo hprt* mutations and of the differentiation stage *in vivo* at which the *hprt* mutation occurred. The scheme to interpret these comparisons has been described fully (Nicklas *et al.*, 1986; Albertini *et al.*, 1990, 1991). Thus, the approach to RBC mutations is comparable for T lymphocytes: Determination of whether they arise in stem cells or in more differentiated progeny. In T cells, stem cells are of pre-thymic origin; the more differentiated cells are mature, post-thymic T cells. In adults, *in vivo hprt* mutations in T cells occur predominantly in mature, differentiated (post-thymic) cells (Nicklas *et al.*, 1989). By contrast, placental blood *hprt* mutant isolates frequently show a pattern that indicates pre-thymic (stem cell) mutations (McGinniss *et al.*, 1989).

A great diversity of TCR gene rearrangements can be identified by Southern blot analyses. In wild type (non-mutant) T-cell clones, TCR gene rearrangements are very rarely shared. In a study of ~20 wild type clones in each of three individuals, no TCR defined sets were observed (Nicklas *et al.*, 1989); while in a much larger study (339 wild type clones from one individual), only three pairs of clones were seen (99.1% unique clones). By contrast, TCR gene pattern sharing is not uncommon among *hprt* mutant isolates (~10% of mutants from the same individual share patterns). This observation, along with the finding that different *hprt* changes are often seen among mutant isolates with the same TCR gene rearrangement patterns, suggests that background spontaneous *in vivo hprt* mutations in T cells arise preferentially in actively dividing cells (Nicklas *et al.*, 1989).

The finding that 90% of *hprt* mutants suggests unique TCR gene patterns argues that most measured Mfs, at least in normal young men with normal Mf values, closely approximate mutation frequencies. However, individuals with very high

hprt T-cell Mf values may show large discordances between the frequencies of mutants and the frequencies of the underlying mutations. Significantly, TCR gene analyses often show remarkable clonality as defined by TCR gene rearrangements among the mutants. Nicklas *et al.* (1988) reported an Mf value in an otherwise normal individual that had risen progressively over 4 years to approximately 10^{-3} (100-fold above background). More than 98% of these mutant isolates (from hundreds analyzed) showed the same TCR gene rearrangement pattern. Furthermore, molecular analyses of *hprt* revealed that *hprt* mutations had occurred repetitively in this clone, including secondary *hprt* mutations in cells that had already suffered primary *hprt* mutations. This observation of sequential somatic mutations occurring over time in an *in vivo* dividing T-cell clone is the first of its kind. This observation further supports the hypothesis that spontaneous *hprt* mutations arise preferentially *in vivo* in dividing T cells.

Despite abundant information on the *hprt* system, the representative nature of the *hprt* gene for mutational studies has been questioned, because its location on the X-chromosome makes it, therefore, either physically or functionally haploid in all individuals. Detection of gene conversion or homologous crossing-over events at a haploid locus is impossible. Therefore, potentially important events may go undetected, unless *in vivo* mutations can be measured at an autosomal locus.

HLA mutations

The HLA complex includes several linked loci located on the short arm of chromosome 6. These loci contain two classes of genes encoding cell surface recognition (restriction) molecules of importance in immune responses (Bodmer, 1984). The class I loci contain the HLA-A, HLA-B, and HLA-C genes, and a large number of as yet undefined genes. Class R (HLA-DP, HLA-DQ, HLA-ĐP), and class III loci (complement genes) are also present.

The HLA system is extremely polymorphic. For class I genes, at least 23 alleles of HLA-A, 47 alleles of HLA-B, and eight alleles of HLA-C are known; they are co-dominantly expressed in heterozygous individuals (Bodmer, 1984). Loss due to mutation of an antigen specified by one allele can be easily detected.

Studies of HLA mutations arising in virus transformed B lymphocytes *in vitro* have shown that such mutations occur (Pious *et al.*, 1973; Pious *et al.*, 1977; Kavathas *et al.*, 1980a, 1980b; Gladstone *et al.*, 1982; Nicklas *et al.*, 1984). Detection of mutants involves immunoselection using specific anti-HLA sera or monoclonal antibodies plus complement. This method has been applied to T lymphocytes to study *in vivo* HLA mutation using a cloning assay, similar to that described for studies of *hprt* mutations (Janatipour *et al.*, 1988).

Presently, published reports of *in vivo* HLA mutations have come from a group studying mutations of the class I HLA-A gene (Janatipour *et al.*, 1988; Turner *et al.*, 1988; McCarron *et al.*, 1989). This gene contains seven exons and spans 5 kb. Although initial results suggested that HLA Mfs were different for the two HLA-A

alleles studied (i.e., HLA-A2 and HLA-A3), subsequent improvement in the immunoselection method resolved this discrepancy. Mean Mf values for both alleles for 21 normal adults, aged 18 to 50 years, were essentially the same at 30×10^{-6} ($\pm$ 1 SD = 14.8×10^{-6}). Comparable Mf values for 10 older individuals, aged 70 to 90 years, were significantly higher at 71.6×10^{-6} ($\pm$ 40.6×10^{-6}) (McCarron *et al.*, 1989). Therefore, background HLA Mf values are approximately five- to 10-fold higher than are background GPA and *hprt* Mf values, with an age effect being observed.

HLA mutant T-cell isolates have been studied for DNA changes. Complete deletions of the region of the HLA gene being probed were reported for approximately 30% of the *in vivo* background mutants (Tumer *et al.*, 1988). Some deletions were quite large as demonstrated by the associated loss of the HLA-B allele in the *cis* position to the deleted HLA-A allele (approximately 1000 kb distant). The most recent molecular analyses of *in vivo* derived HLA loss mutants by Southern blot studies show that approximately 30% resulted from mitotic recombination (Morley *et al.*, 1990). Therefore, this important class of mutational events is detectable.

Thus far, the reports of TCR gene rearrangement patterns among HLA mutants from single individuals have been insufficient to determine the T-cell differentiation stage for *in vivo* mutations or clonality, similar to what has been found in the *hprt* system. No HLA studies have been reported in newborns or in individuals with DNA repair deficiency syndromes. However, as this assay becomes operational, further studies are certain to follow.

9.3 OTHER MEASURES OF *IN VIVO* SOMATIC CELL GENE MUTATION

The four assays thus far considered form our current frame of reference regarding *in vivo* somatic cell gene mutations in humans. However, new systems are under development. For example, detection of rare RBCs in peripheral blood that express the Tn antigen has been proposed as a measure of somatic mutation in erythroid precursor cells (Bigbee *et al.*, 1990b). Expression of the Tn antigen apparently results from loss of 3-6-D-galactosyltransferase activity, which normally glycosylates the Tn structure to a product that eventually becomes the M or N blood group antigen (Bigbee *et al.*, 1990b). Rare individuals exist whose blood contains large numbers of Tn RBCs. Some of these individuals also have hemolytic anemia. The disease condition is thought to arise from somatic cell mutation(s) in one or more RBC precursors, with subsequent amplification of these stem cell mutants (Bigbee *et al.*, 1990b). If so, this situation illustrates the complex relationships between mutants and their underlying mutations that will be encountered as each new marker of *in vivo* somatic cell mutations is studied.

New markers may soon be available for the T-cell assays. Diphtheria toxin-resistant mutant T-cells arising *in vivo* in humans have been described, and this

system may be developed for possible use in mutagenicity testing (Albertini, 1982). Losses and alterations of TCR genes, occurring *in vivo* in humans have been described (Kyoizumi *et al.*, 1990). These effects may result from somatic mutations, and might be developed as mutagenicity markers. Also, *in vivo* mutation studies with lymphocytes need not be restricted to T cells. Some investigators have reported *in vivo hprt* mutations in both peripheral blood B lymphocytes and in natural killer (NK) cells in an atomic bomb survivor (Hakoda *et al.*, 1989b). This report illustrates that such systems can probably be developed into useful assays, and is of interest also because it again demonstrates the complex relationship between mutants arising *in vivo* and their underlying mutations. An original *hprt* mutation in a lymphoid stem cell in an atomic bomb survivor was amplified during differentiation, eventually appearing in T lymphocytes (with multiple TCR gene rearrangement patterns), in B lymphocytes (with multiple immunoglobulin gene rearrangement patterns), and in NK cells.

Despite the progress with cell growth mutation systems, such methods have inherent limitations. All methods based on cell growth, such as the cloning assays, employ selective systems to detect and isolate rare mutants. Selection itself may restrict the numbers and kinds of mutants that can be studied (Bradley, 1980, 1983; Worton and Grant, 1985). The lymphocyte assays use selective conditions that kill non-mutant cells, and allow the appropriate mutants to survive. However, mutations that do not alter the mutant molecule sufficiently to resist the selective agent will not allow the cell to survive, thus eliminating this class of mutations from recognition. The red cell assays, while not selecting against non-mutant cells in this way, employ immunological methods for mutant recognition. Therefore, mutations that produce mutant molecules differing from non-mutant molecules at sites other than the antibody recognition site may also be undetected. Since different monoclonal antibodies against the same cell marker may differ with respect to epitope recognition, different spectra of mutations may be recognized when different antibodies are used. Mixtures of monoclonal antibodies, or antisera, may be expected to produce mutational spectra that contain a large proportion of deletions.

Some agreement exists that definitions of *in vivo* mutational spectra at the DNA level will help to define the contribution of environmental mutagens to the overall burden of somatic mutations in humans. Attempts are under way to identify molecular spectra that underlie both the background and induced *in vivo* mutations, with the expectation that at least some mutagens will give characteristic spectra that may serve as signatures for exposures to an agent. The cell-by-cell approach may be inadequate to describe mutational spectra, because the methods lack sensitivity, are laborious, and distort these spectra through selection. Finally, all cell growth based methods require that the cells being analyzed have the ability to propagate *in vitro* in order to provide sufficient DNA or RNA for analysis.

To overcome these limitations, DNA-based methods which rely on denaturing gradient gel electrophoresis and high fidelity DNA amplification using the PCR are being proposed and developed for *in vivo* mutagenicity studies (Cariello *et al.*,

1988; Keohavong and Thily, 1991). The methods envisaged will not employ selection for mutant cells in order to avoid distortions, and will use genetic elements present in multiple copies per cell to achieve high sensitivity. Although only artificial reconstruction experiments have been described thus far, enormous technical difficulties have been overcome (Cariello *et al.*, 1988; Keohavong and Thilly, 1991). Certain technical problems still remain, and some means must be devised to account for clonal amplification of mutants (i.e., the mutant/mutation relationship) when describing mutational spectra. This innovative method clearly addresses limitations of current cell-based systems, but is a system under development.

Although this review is concerned with somatic cell gene mutations *in vivo* in humans, in principle, all markers and their analogs used to assess somatic mutations *in vivo* in humans are available for use in animals. The further development of such *in vivo* mutation assays in animals is important for several reasons. Some basic studies can be done only in animals, and a variety of investigations can be carried out, including those defining dose-response relationships. These are necessary to fully interpret the results of human *in vivo* studies. This area is an important one for further research.

Several groups have now reported results of studies of T-cell *hprt* mutations arising in animals (Gocke *et al.*, 1983; Jones *et al.*, 1985; Inamizu *et al.*, 1986; Dempsey and Morley, 1986; Ward *et al.*, 1989; Ammenheuser *et al.*, 1990; Aidoo *et al.*, 1991; Zimmer *et al.*, 1991). Both a short-term autoradiographic and a cloning assay have been used to study these *in vivo* events. Both are widely used in humans, and a large database has been accumulated for these *in vivo* mutations. The background mutant frequency values have thus far been determined for mouse, rat, and non-human primates, and are essentially the same as for humans. The molecular bases of the *in vivo* mutations in animals are being investigated, in hope that they will be applicable to humans. Results of molecular analyses of the *hprt* changes have already been described for mutations in mouse and monkey (Jones *et al.*, 1987; Burkhart-Schultz *et al.*, 1990; Harback *et al.*, 1991). The sequence analysis of spontaneous and *N*-ethyl-*N*-nitrosourea-induced mutations arising *in vivo* in the cynomologus monkey have been described (Harback *et al.*, 1991); similar studies are under way in the rat. Induced mutations also are being studied in animals. Elevated mutant frequencies have been reported for smoking baboons (Ammenheuser *et al.*, 1990), as has the time course for appearance and persistence of N-ethyl-N-nitrosourea-induced *hprt* mutants in Cynomologus monkeys (Zimmer *et al.*, 1991). The animal systems clearly respond to model environmental mutagens.

An assay described for *in vivo* somatic cell mutations in animals is the granuloma pouch assay in rodents. It allows measurement of *hprt* or other mutations in the fibroblasts of specially developed subcutaneous pouches in rats. Quantitative studies of induced mutation are possible, and mutant cells are available for molecular analyses.

9.4 CONCLUSIONS

This past decade has seen significant advances in our ability to detect and analyze *in vivo* somatic cell gene mutations in humans. The four assays considered here have demonstrated conclusively that such mutations occur; however, the underlying biology of these events has not been fully studied.

One factor of importance for *in vivo* somatic cell gene mutations will probably be the differentiation stage at which they occur. This stage can be determined with current methods. The long memory of the GPA RBC assay for atomic bomb survivors can be contrasted with the short memory of this same assay for cancer patients after chemotherapy. The former presumably indicates *in vivo* mutation in pluripotent stem cells, the latter mutation expressed in more differentiated progeny. The differentiation stage of mutation is being studied by TCR gene probes in lymphocytes. Lymphocyte *hprt* mutations in the human fetus often arise in pre-thymic stem cells, whereas these same mutations appear to arise in differentiated, post-thymic T lymphocytes of adults. Lymphocyte mutations in the fetus, therefore, may have considerably more biological importance than similar mutations in the adult. If multiple somatic hits are important in disorders such as cancer, one way to increase the probability that multiple mutations will eventually converge in a single mutant cell is to have some of these events occur early, when few cells exist.

A mechanism that can result in multiple mutations occurring in a single mutant cell in adults is unremitting *in vivo* cell division, that creates space for sequential gene mutations to accumulate in descendants of the same cell. If this is one route to malignant transformation, perhaps the evolution of cancer can occur by spontaneous mutations with the role of environmental toxicants being primarily to induce this unremitting cell division. This recognition of the role of cell division and the accumulation of multiple mutations in individual mutant cells in T lymphocytes may allow insights into the evolution of a number of somatic genetic disorders. Whether or not this phenomenon is unique to lymphocytes remains to be seen.

Both the GPA and the HLA assays have demonstrated somatic crossing-over or gene conversion *in vivo* in human somatic cells. These processes can now be studied with *in vivo* model systems in humans. An understanding of their role in pathological processes and in differentiation will certainly be facilitated by the availability of these systems.

The final role of human *in vivo* gene mutation assays for toxicology remains to be defined. The low background Vf and Mf values for the four assays permit a baseline for assessing environmental effects. However, the relative rank-order of these values (i.e., HbS Vf < GPA Vf = *hprt* Mf < HLA Mf) is explained only partially. The agreement of GPA Vf with *hprt* Mf might be expected by the similar sizes of these two genes, but not by the different kinetics through which mutants arise. The reasons for the higher HLA Mf values are unknown, but may relate also to different kinetics or lack of *in vivo* selection. However, immunological selection

in vivo might be expected for mutant cells bearing other than null surface marker mutations. Very frequent *in vivo* crossing-over events could explain the relatively high HLA Mf values; but this occurrence should apply also to GPA homozygous variants. The GPA and *hprt* assays, have been used to study individuals with DNA repair defects, and the expected increases in mutants have been observed; yet this effect was not seen in studies using the HbS system.

Three assays have thus far been used to study individuals exposed to a variety of environmental mutagens. Mean elevations in Vf and Mf values have usually been observed, with a good deal of interindividual variability. This phenomenon suggests that the assays may be useful as indicators of population exposures to environmental mutagens, but leaves unresolved the question of their utility for making assessments of exposures and their health consequences. In terms of sensitivity, the GPA and the *hprt* assays have demonstrated their ability to detect the mutagenic effects of smoking; and all of the mutation detection assays clearly record increases in Mf or Vf with increasing age.

Data on which to base a toxicologic evaluation of these *in vivo* mutagenicity assays are rapidly accumulating. However, given the biological considerations, perhaps the effects of mutagen exposures on *in vivo* somatic mutations will not be reflected in terms as simple as quantitative dose–responses. Definition of mutational spectra may be required to define environmental genotoxic effects, at least following acute exposures before cell division confounds the situation. Clearly, much research is still needed, and the tools for this task are now in hand.

9.5 REFERENCES

Aidoo, A., Lyn-Cook, L.E., Heflich, R.H., and Casciano, D.A. (1991) Development of an assay for measuring 6-thioguanine-resistant lymphocytes induced *in vivo* in the rat. *Environ. Mol. Mutagen.* **17**, 6.

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

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

Albertini, R.J., Castle, K.L., and 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., Gannett, I.N., Lambert, B., Thilly, W.G., and Vrieling, H. (1989) Mutation at the *hprt* locus: report of a workshop. *Mutat. Res.* **216**, 65–88.

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

Albertini, R.J., O'Neill, J.P., Nicklas, J.A., Allegretta, M., Recio, L., and Skopek, T.R. (1991) Molecular and clonal analysis of *in vivo hprt* mutations in human cells. In: Garner, R.C., Farmer, P.B., Steel, G.T., and Wright, A.S. (Eds) *Human Carcinogen Exposure: Biomonitoring and Carcinogen Risk Assessment*, pp. 103–126. Oxford University Press,

Oxford.

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

Ammenheuser, M.M., Ward, J.B., Au, W.W., and Belli, J.A. (1989) A prospective study comparing 6-thioguanine-resistant variant frequencies with chromosome aberration frequencies in lymphocytes from radiotherapy and chemotherapy patients. *Environ. Mol. Mutagen.* **14**, 9.

Ammenheuser, M.M., Ward, J.B., Jr., Whorton, E.B., Jr., and Marshall, M.V. (1990) A longitudinal study comparing premolding and postmolding 6-thioguanine-resistant variant frequencies in baboons trained to smoke cigarettes. *Environ. Mol. Mutagen.* **15**, 6.

Ammenheuser, M.M., Au, W.W., Whorton, E.B., Jr., Belli, J.A., and 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.

Amneus, H. and Eriksson, L. (1986) The frequency of 6-thioguanine-resistant human peripheral blood lymphocytes as determined by flow cytometry and by clonal propagation. *Mutat. Res.* **173**, 61–66.

Amneus, H., Matsson, P., and Zetterberg, G. (1982) Human lymphocytes resistant to 6-thioguanine: restrictions in the use of a test for somatic mutations arising *in vivo* studied by flow-cytometric enrichment of resistant cell nuclei. *Mutat. Res.* **106**, 163–178.

Amneus, H., Sjogren, B., and Zetterberg, G. (1984) Resistance to 6-thioguanine in spontaneously cycling and in mitogen-stimulated human peripheral lymphocytes. *Mutat. Res.* **139**, 41–44.

Atwood, K.C. and Petter, F.J. (1961) Erythrocyte automosaicism in some persons of known genotype. *Science* **134**, 2100–2102.

Atwood, K.C. and Scheinberg, S.L. (1958) Somatic variation in human erythrocyte antigens. *J. Cell Comp. Physiol.* **52**, 97–123.

Bigbee, W.L., Branscomb, E.W., and Jensen, R.H. (1983) Counting of RBC variants using rapid flow techniques. In: de Serres, F.J. and Sheridan, W. (Eds) *Environmental Science Research, Utilization of Mammalian Specific Locus Studies in Hazard Evolution and Estimation of Genetic Risk*, Vol. 28, pp. 39–54, Plenum Press, New York.

Bigbee, W.L., Langlois, R.G., Swift, M., and Jensen, R.H. (1989) Evidence for an elevated frequency of *in vivo* somatic cell mutations in ataxia telangiectasia. *Am. J. Hum. Genet.* **44**, 402–408.

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

Bigbee, W.L., Langlois, R.G., Stanker, L.H., Vanderlaan, M., and Jensen, R.H. (1990b) Flow cytometric analysis of erythrocyte populations in Tn syndrome blood using monoclonal antibodies to glycophorin A and the Tn antigen. *Cytometry* **11**, 261–271.

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

Bradley, W.E.C. (1980) Conditional instability of induced variants of CHO. Transient sensitivity to contact with wild-type cells. *Exp. Cell Res.* **129**, 251–263.

Bradley, W.E.C. (1983) Mutation at autosomal loci of Chinese hamster ovary cells: Involvement of a high-frequency event silencing two linked genes. *Mol. Cell. Biol.* **3**, 1172–1181.

Bradley, W.E.C., Gareau, J.L.P., Seifert, A.M., and Messing, K. (1987) Molecular characterization of 15 rearrangements among 90 human *in vivo* somatic mutants shows that deletions predominate. *Mol. Cell. Biol.* **7**, 956–960.

Burkhart-Schultz, K., Strout, C.L., and Jones, L.M. (1990) Mouse model for somatic mutation at the *hprt* gene: molecular and cellular analyses. *Prog. Clin. Biol. Res.* **340C**, 5–14.

Cariello, N.F., Scott, J.K., Kat, A.G., Thilly, W.G., and Keohavong, P. (1988) Resolution of a missense mutant in human genomic DNA by denaturing gradient gel electrophoresis and direct sequencing using *in vitro* DNA amplification: hprt. *Am. J. Hum. Genet.* **42**, 726–734.

Cole, J., Green, M.H.L., James, S.E., Henderson, L., and Cole, H. (1988) A further assessment of factors influencing measurements of thioguanine-resistant mutant frequency in circulating T-lymphocytes. *Mutat. Res.* **204**, 493–507.

Craft, T.R., Cariello, N.F., and Skopek, T.R. (1991) Mutational profile of the human *hprt* gene: Summary of mutations observed to date. *Environ. Mol. Mutagen.* **17**, 18.

Deisseroth, A., Nienhuis, A., Turner, P., Velez, R., Anderson, W.F., Ryddle, F., Lawrence, J., Creagan, R., and Kucherlapati, R. (1977) Localization of the human alpha-globin structural gene to chromosome 16 in somatic cell hybrids by molecular hybridization assay. *Cell* **12**, 205–218.

Deisseroth, A., Nienhuis, A., Lawrence, J., Giles, R., Tumer, P., and Ruddle, F.H. (1978) Chromosomal location of human beta-globin gene on human chromosome 11 in somatic cell hybrids. *Proc. Natl Acad. Sci. USA* **75**, 1456–1460.

Dempsey, J.L., and Morley, A.A. (1986) Measurement of *in vivo* mutant frequency in lymphocytes in the mouse. *Environ. Mutagen.* **8**, 385–391.

Dempsey, J.L., Seshadri, R.S., and Morley, A.A. (1985) Increased mutation frequencey following treatment with cancer chemotherapy. *Cancer Res.* **45**, 2873–2877.

DuPont, B.R., Bigbee, W.L., Grant, S.G., Jensen, R.H., and Langlois, R.G. (1991) Molecular analysis of glycophorin A variant reticulocytes. *Environ. Mol. Mutagen.* **17**, 23.

Edwards, A., Voss, H., Rice, P., Civitello, A., Stegemann, J., Schwager, C., Zimmerman, J., Erfle, H., Caskey, C.T., and Ansorge, W. (1990) Automated DNA sequencing of the human *hprt* locus. *Genomics* **6**, 593–608.

Furthmayer, H. (1977) Structural analysis of a membrane glycoprotein: Glycophorin A. *J. Supramol. Struc.* **7**, 121–134.

Fuscoe, J.C., Zimmerman, L., O'Neill, J.P., Nicklas, J., and Albertini, R. (1991a) Molecular analysis of deletion breakpoints within the *hprt* gene of T lymphocytes from human newborns. *Environ. Mol. Mutagen.* **17**, 25.

Fuscoe, J.C., Zimmerman, L., and Albertini, R.J. (1991b) Molecular analysis of *in vivo hprt* deletion mutations in human cells: PCR and DNA sequencing. *Environ. Mol. Mutagen.* **17**, 84.

Gahmberg, C.G., Jaidnen, M., and Andersson, L.C. (1979) Expression of the major red cell sialoglycoprotein, glycophorin A, in the human leukemic cell line K562. *J. Biol. Chem.* **254**, 7442–7448.

Gladstone, P., Fueresz, L., and Pious, D. (1982) Gene dosage and gene expression in the HLA region: Evidence from deletion variants. *Proc. Natl Acad. Sci. USA* **79**, 1235–1239.

Gocke, E., Eckhardt, E., King, M.T., and Wild, D. (1983) Autoradiographic detection of 6-thioguanine-resistant lymphocytes of mice: A novel system in somatic mutagenicity testing. *Mutat. Res.* **113**, 455–465.

Hakoda, M., Aidyama, M., Kyoizumi, S., Kobuke, K., Awa, A.A., and Yamaiddo, M. (1988)

Measurement of *in vivo* HGPRT-deficient mutant cell frequency using a modified method for cloning human peripheral blood T lymphocytes. *Mutat. Res.* **197**, 161–169.

Hakoda, M., Hirai, Y., Kyoizumi, S., and Aldyama, M. (1989a) Molecular analyses of *in vivo hprt* mutant T-cells from atomic bomb survivors. *Environ. Mol. Mutagen.* **13**, 25–33.

Hakoda, M., Hirai, Y., Shimba, H., and Kusunold, Y. (1989b) Cloning of phenotypically different human lymphocytes originating from a single stem cell. *J. Exp. Med.* **169**, 1265–1276.

Harback, P.R., Filipunas, A.L., Zimmer, D.M., and Aaron, C.S. (1991) DNA sequence analysis of spontaneous and *N*-ethyl-*N*-nitroso-urea-induced mutations arising *in vivo* in Cynomolgus monkey. *Environ. Mol. Mutagen.* **17**, 29–30.

Henderson, J.F., Kelley, W.N., Rosenbloom, F.M., and Seegmutter, J.E. (1969) Inheritance of purine phosphoribosyltransferase in man. *Am. J. Hum. Genet.* **21**, 61–70.

Inamizu, T., Kinohara, N., Chang, M.P., and Maldnodan, T. (1986) Frequency of 6-thioguanine-resistant T cells is inversely related to the declining T cell activities in aging mice. *Proc. Natl Acad. Sci. USA* **83**, 2488–2491.

Janatipour, M., Trainor, K.J., Kutlaca, R., Bennett, G., Hay, J., Tumer, D.R., and Morley, A.A. (1988) Mutations in human lymphocytes studied by an HLA selection system. *Mutat. Res.* **198**, 221–226.

Jensen, R.H., Bigbee, W.L., and Langlois, R.G. (1987) *In vivo* somatic mutations in the Glycophorin A locus of human erythroid cells. In: Moore, M.M., DeMarini, D.M., deSerres, FJ., and Tindall, K.R. (Eds) *Mammalian Cell Mutagenesis*, Banbury Report 28, pp. 149–159. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Jones, I.M., Burkhart-Schultz, K.,.and Carrano, A.V. (1985) A study of the frequency of sister-chromatid exchange and of thioguanine-resistant cells in mouse spleen lymphocytes after *in vivo* exposure to ethylnitrosourea. *Mutat. Res.* **143**, 245–249.

Jones, I.M., Burkhart-Schultz, K., and Crippen, T.L. (1987) Cloned mouse lymphocytes permit analysis of somatic mutations that occur *in vivo*. *Somat. Cell. Mol. Genet.* **13**, 325–333.

Kavathas, P., Bach, F.H., and DeMars, R. (1980a) Gamma ray-induced loss of expression of HLA and glyoxalase I alleles in lymphoblastoid cells. *Proc. Natl Acad. Sci. USA* **77**, 4251–4255.

Kavathas, P., DeMars, R., and Bach, F.H. (1980b) Hemizygous HLA mutants of lymphoblastoid cells: A new cell type for histocompatibility testing. *Hum. Immunol.* **1**, 317–324.

Keohavong, P. and Thilly, W.G. (1991) Analysis of mutational spectra in human cells. *Environ. Mol. Mutagen.* **17**, sl9–36.

Kudo, S. and Fukuda, M. (1989) Structural organization of glycophorin A and B genes: Glycophorin B gene evolved by homologous recombination at Alu repeat sequences. *Proc. Natl Acad. Sci. USA* **86**, 4619–4623.

Kyoizumi, S., Nakamura, N., Takebe, H., Tatsumi, K., German, J., and Aldyama, M. (1989a) Frequency of variant erythrocytes at the glycophorin-A locus in two Bloom's syndrome patients. *Mutat. Res.* **214**, 215–222.

Kyoizumi, S., Nakamura, N., Hakoda, M., Awa, A.A., Bean, M.A., Jensen, R.H., and Aldyama, M. (1989b) Detection of somatic mutations at the glycophorin A locus in erythrocytes of atomic bomb survivors using a single beam flow sorter. *Cancer Res.* **49**, 581–588.

Kyoizumi, S., Aidyama, M., Hirai, Y., Kusunold, Y., Umeld, S., Nakamura, N., Endoh, K., Konishi, J., Sasaid, M.S., and Mori, T. (1990) Frequency of mutant T lymphocytes

defective in the expression of T cell antigen receptor gene among radiation exposed people. Radiation Effects Research Laboratory Technical Reports.

Langlois, R.G., Bigbee, W.L., Jensen, R.H., and German, J. (1989a) Evidence for increased *in vivo* mutations and somatic recombination in Bloom's syndrome. *Proc. Natl Acad. Sci. USA* **86**, 670–674.

Langlois, R.G., Nisbett, B., Bigbee, W.L., and Jensen, R.H. (1989b) An improved flow cytometric assay for mutations at the Glycophorin A locus in humans. *Cytometry* **11**, 513–521.

Langlois, R.G., Bigbee, W.L., and Jensen, R.H. (1990) The Glycophorin A assay for somatic cell mutations in humans. In: Mendelson, M.L. and Albertini, R.J. (Eds) *Mutation and the Environment Part C: Somatic and Heritable Mutation, Adduction, and Epidemiology: Progress in Clinical and Biological Research* **340C**, pp. 47–56. Wiley-Liss, New York.

Langlois, R.G., DuPont, B.R., Bigbee, W.L., Grant, S.G., and Jensen, R.H. (1991) Development of an assay method for *in vivo* somatic cell mutations detected in human reticulocytes. *Environ. Mol. Mutagen.* **17**, s 19, 41.

Lippert, M.J., Manchester, D.K., Hirsch, B., Nicklas, J.A., O'Neill, J.P., Sweet, L.M., and Albertini, R.J. (1990) Pattems of *hprt* mutations detected in newborns and elderly by cloning assay. *Environ. Mol. Mutagen.* **15**, s 17, 35.

Matsson, P., Amneus, H., Djupsjobacka, M., Eriksson, B., Eriksson, L., Felner-Feldegg, H., and Zetterberg, G. (1985) Direct and indirect flow cytometric enumeration of 6-thioguanine-resistant human peripheral blood lymphocytes. *Cytometry* **6**, 648–656.

McCarron, M.A., Kutlaca, A., and Morley, A.A. (1989) The HLA-A mutation assay: Improved technique and normal results. *Mutat. Res.* **225**, 189–193.

McGinniss, M.J., Falta, M.T., Sullivan, L.M., and Albertini, R.J. (1990) *In vivo hprt* mutant frequencies in T-cells of normal human newboms. *Mutat. Res.* **240**, 117–126.

Mendelsohn, M.L. (1991) Genetic toxicology of the human: The current status of somatic gene mutation. In: Garner, R.C., Farmer, P.B., Steel, G.T., and Wright, A.S. (Eds) *Biomonitoring and Risk Assessment*. Oxford University Press, Oxford.

Messing, K. and Bradley, W.E.C. (1985) *In vivo* mutant frequency rises among breast cancer patients after exposure to high doses of gamma radiation. *Mutat. Res.* **152**, 107–112.

Messing, K., Seifert, A.M., and Bradley, W.E.C. (1986) *In vivo* mutant frequency of technicians professionally exposed to ionizing radiation. In: Sorsa, M. and Norppa, H. (Eds) *Monitoring of Occupational Genotoxicants*, pp. 87–97. Alan R. Liss,Inc., New York.

Morley, A.A., Trainor, K.J., Seshadri, R., and Ryan, R.G. (1983) Measurement of *in vivo* mutations in human lymphocytes. *Nature* **302**, 155–156.

Morley, A.A., Trainor, K.J., Dempsey, J.L., and Seshardi, R.S. (1985) Methods for study of mutations and mutagenesis in human lymphocytes. *Mutat. Res.* **147**, 363–367.

Morley, A.A., Grist, S.A., Turner, D.R., Kutlaca, A., and Bennett, G. (1990) Molecular nature of *in vivo* mutations in human cells at the autosomal HLA-A locus. *Cancer Res.* **50**, 4594–4587.

Nicklas, J.A., Kfiyachi, J., Taurog, J.D., Wee, S.L., Chen, L.K., Grumet, F.C., and Bach, F.H. (1984) HLA loss variants of a B27+ lymphoblastoid cell line: Genetic and cellular characterization. *Hum. Immunol.* **11**, 19–30.

Nicklas, J.A., O'Neill, J.P., and Albertini, R.J. (1986) Use of T cell receptor gene probes to quantify the *in vivo hprt* mutations in human T-lymphocytes. *Mutat. Res.* **173**, 65–72.

Nicklas, J.A., O'Neill, J.P., Sullivan, L.M., Hunter, T.C., Allegretta, M., Chastenay, B.F., Libbus, B.L., and Albertini, R.J. (1988) Molecular analyses of *in vivo* Hypoxanthine-guanine phosphoribosyltransferase mutations in human T lymphocytes: II.

Demonstration of a clonal amplification of *hprt* mutant T lymphocytes *in vivo*. *Environ. Mol. Mutagen.* **12**, 271–294.

Nicklas, J.A., Hunter, T.C., O'Neill, J.P., and Albertini, R.J. (1989) Molecular analyses of *in vivo hprt* mutations in human T-lymphocytes: III. Longitudinal study of *hprt* gene structural alterations and T cell clonal origins. *Mutat. Res.* **215**, 147–160.

Nicklas, J.A., Falta, M.T., Hunter, T.C., O'Neill, J.P., Jacobson-Kram, D., Williams, J., and Albertini, R.J. (1990) Molecular analysis of *in vivo hprt* mutations in human lymphocytes V. Effects of total body irradiation secondary to radioimmunoglobulin therapy (RIT). *Mutagenesis* **5**, 461–468.

Nicklas, J.A., Hunter, T.C., O'Neill, J.P., and Albertini, R.J. (1991) Fine structure mapping of the *hprt* region of the human X chromosome (Xq26) *Am. J. Hum. Genet.* **49**, 267–278.

O'Neill, J.P., McGinniss, M.J., Berman, J.K., Sullivan, L.M., Nicklas, J.A., and Albertini, R.J. (1987) Refinement of a T-lymphocyte cloning assay to quantify the *in vivo* thioguanine-resistant mutant frequency in humans. *Mutagenesis* **2**, 87–94.

Ostrosky-Wegman, P., Monters, R., Gomez, M., and Cortinas de Nova, C. (1987) 6-Thioguanine resistant T lymphocyte determination as a possible indicator of radiation exposure. *Environ. Mutagen.* **9**, 81.

Papayannopoulou, T.H., Brice, M., and Stamatoyannopoulos, G. (1977a) Hemoglobin F synthesis *in vitro:* Evidence for control at the level of primitive erythroid stem cells. *Proc. Natl Acad. Sci. USA* **74**, 2923–2927.

Papayannopoulou, T.H., Nute, P.E., Stamatoyannopoulos, G., and McGuire, T.G. (1977b) Hemoglobin ontogenesis: Test of the gene exclusion hypothesis. *Science* **197**, 1215–1216.

Patel, P.I., Framson, P.E., Caskey, C.T., and Chinault, A.C. (1986) Fine structure of the human hypoxanthine phosphoribosyltransferase gene. *Mol. Cell. Biol.* **6**, 393–403.

Pious, D., Hawley, P., and Forrest, G. (1973) Isolation and characterization of HLA variants in cultured human lymphoid cells. *Proc. Natl Acad. Sci. USA* **70**, 1397–1400.

Pious, D., Soderland, C., and Gladstone, P. (1977) Induction of HLA mutations by chemical mutagens in human lymphoid cells. *Immunogenesis* **4**, 437–488.

Recio, L., Cochrane, J., Simpson, D., Skopek, T.R., O'Neill, J.P., Nicklas, J.A., and Albertini, R.J. (1990) DNA sequence analysis of *in vivo hprt* mutation in human T-lymphocytes. *Mutagenesis* **5**, 505–510.

Rossi, A., Thijssen, J., Tates, A., Vrieling, H., and Natarajan, A. (1990) Mutations affecting RNA splicing in man are detected more frequently in somatic than in germ cells. *Mutat. Res.* **244**, 353–357.

Russell, L.B. and Major, M.H. (1957) Radiation induced presumed somatic mutants in the house mouse. *Genetics* **42**, 161–175.

Sala-Trepat, M., Cole, J., Green, M. H., Rigaud, O., *et al.* (1990) Genotoxic effects of radiotherapy and chemotherapy on the circulating lymphocytes of breast cancer. III: Measurement of mutant frequency to 6-thioguanine resistance. *Mutagenesis* **5**, 593–598.

Sanderson, B.J., Dempsey, J.L., and Morley, A.A. (1984) Mutations in human lymphocytes: Effects of X- and UV-irradiation. *Mutat. Res.* **140**, 223–227.

Seifert, A.M., Bradley, W.C., and Messing, K. (1987) Exposure of nuclear medicine patients to ionizing radiation is associated with rises in HPRT-mutant frequency in peripheral T-lymphocytes. *Mutat. Res.* **191**, 57–63.

Stamatoyannapoulos, G., Wood, W.G., Papayannapoulou, T.N., and Nute, P.E. (1975) An atypical form of hereditary persistence of fetal hemoglobin in blacks and its association with sickle cell trait. *Blood* **46**, 683–692.

Stamatoyannapoulos, G., Nute, P., Lindsley, D., Farguhar, M.B., Nakamato, B., and

Papayannepoulou, T.H. (1984) Somatic-cell mutation monitoring system based on human hemoglobin mutants. In: Ansari, A.A. and deSerres, F.J. (Eds) *Single Cell Monitoring Systems, Topics in Chemical Mutagenesis*, Vol. 3, pp. 1–35, Plenum Press, New York.

Stark, M.H., Tucker, J.H., Thomson, E.J., and Perry, P.E. (1984) An automated image analysis system for the detection of rare autoradiographically labeled cells in the human lymphocyte HGPRT assay. *Cytometry* **5**, 250–257.

Strauss, G.H. and Albertini, R.J. (1979) Enumeration of 6-thioguanine resistant peripheral blood lymphocytes in man as a potential test for somatic mutations arising *in vivo*. *Mutat. Res.* **61**, 353–379.

Sutton, H.E. (1972) In: Sutton, H.E. and Harris, M.I. (Eds) *Mutagenic Effects of Environmental Contaminants*, pp. 121–128. Academic Press, New York.

Sutton, H.E. (1974) Somatic cell mutations. In: Motulsky, A.G., Lenz, W., and Ebling, F.J. (Eds) *Birth Defects: Proceedings of the Fourth International Conference*, Vienna, Austria, 2-8 September, 1973. *Excerpta Medica. Int. Congr. Ser.* **310**, 212–214.

Tates, A.D., Bernini, L.F., Natajaran, A.T., Ploem, J.S., Verwoed, N.P., Cole, J., Green, M.H.L., Arlett, C.F., and Norris, P.N. (1989) Detection of somatic mutations in man: HPRT mutations in lymphocytes and hemoglobin mutations in erythrocytes. *Mutat. Res.* **213**, 73–82.

Turner, D.R., Grist, S.A., Janatipour, M., and Morley, A.A. (1988) Mutations in human lymphocytes commonly involve gene duplication and resemble those seen in cancer cells. *Proc. Natl Acad. Sci. USA* **85**, 3189–3193.

Verwoerd, N.P., Bernini, L.F., Bonnet, J., Tanke, H.J., Natarajan, A.T., Tates, A.D., Sobels, F.H., and Ploem, J.S. (1987) Somatic cell mutations in humans detected by image analysis of immunofluorescently stained erythrocytes. In: Burger, G., Ploem, J.S., and Goerttler, K. (Eds) *Clinical Cytometry and Histometry*, 1st ed., pp. 465–469, Academic Press, San Diego.

Ward, J.B., Ammenhauser, M.M., Morris, D.L., Ramanujam, V.M.S., and Legator, M. S. (1989) Induction of thioguanine resistant mouse lymphocyte variants by subchronic low dose inhalation exposure to benzene. *Environ. Mol. Mutagen.* **14**, 214.

Weatherall, D.J. (Ed) (1985) *The New Genetics and Clinical Practice*, 2nd ed. Oxford University Press, New York.

Wilson, J., Stout, J., Palella, T., Davidson, B., Kelley, W., and Caskey, C.T. (1986) A molecular survey of hypoxanthine-guanine phosphoribosyltransferase deficiency in man. *J. Clin. Invest.* **77**, 188–195.

Wood, W.G., Stamatoyannapoulos, G., Lim, G., and Nute, P.E. (1975) F cells in the adult: Normal values and levels in individuals with hereditary and acquired elevations of HbF. *Blood* **46**, 671–682.

Worton, R.G. and Grant, S.G. (1985) Segregation-like events in Chinese hamster cells. In: Gottesman, M.M. (Ed) *Molecular Cell Genetics*, pp. 831–867, John Wiley and Sons, New York.

Zetterberg, G.H., Amneus, H., and Mattson, P. (1982) Use of flow cytometry to concentrate 6-thioguanine resistant variants of human peripheral blood lymphocytes. In: Bridges, B.A., Butterworth, B.E., and Weinstein, I.B. (Eds) *Indicators of Genotoxic Exposure*, Banbury Report 13, pp. 413–420. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Zimmer, D.M., Aaron, C.S., O'Neill, J.P., and Albertini, R.J. (1991) Enumeration of 6-thioguanine resistant T lymphocytes in the peripheral blood of non-human primates (Cynomolgus monkeys). *Environ. Mol. Mutagen.* **18**, 161–167.