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The Development of *In vitro* Mutagenicity Testing Systems Using T-lymphocytes

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VII. DETAILED PROGRESS REPORT OF DOE SUPPORTED RESEARCH, VCCGL, 1989-1992:

This section will present in detail our progress during the last 3 years of this grant with emphasis on recent work.

1) Results with the *In Vitro* Clonal Assay using the *In Situ* Method

A main aim of this grant has been the development of *in vitro* T-cell mutation assays. Conditions have been defined to measure the *in vitro* induction of mutations at the hypoxanthine-guanine phosphoribosyl transferase (*hprt*) locus in human T-lymphocytes. This assay is a parallel to our *in vivo hprt* assay, in that the same cells are utilized. However, the *in vitro* assay allows for carefully controlled dose response studies.

Since initiation of DOE support, several methods for performing the *in vitro* cloning assay have been developed and reported (O'Neill et al., 1990a, 1991) and studies of radiation exposed cells performed. Molecular analyses have defined the resulting induced mutational spectrum (O'Neill et al., 1990b). In order to quantify *in vitro* induction of mutations at the *hprt* locus in human T-lymphocytes, optimal conditions were defined to allow proliferation of mass cultures for expression of the TG^r phenotype, as well as cell cloning for mutant selection and phenotypic expression time. This has been described in detail in past progress reports.

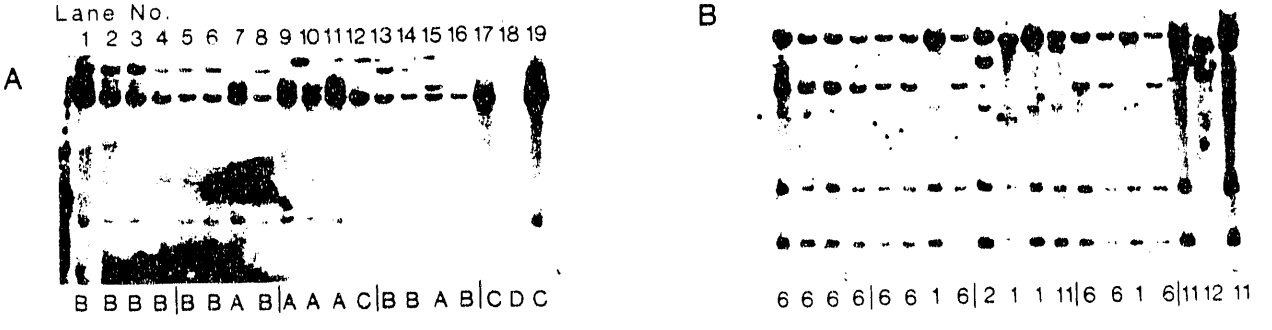


FIGURE 1 - Southern blots of 19 TG^r mutants from exp. P35 cleaved with BamHI and probed with *hprt* (panel A) and a TcRβ (panel B) gene probes. In A), the *hprt* pattern is designated by the letters A-D for each lane. Pattern A is the wild type pattern. The individual in these studies showed the BamHI polymorphism resulting in a 22 kb exon 2+3 fragment and a 12 kb exon 6-9 fragment (Nussbaum et al, 1983). Five of 19 mutants show the normal pattern (lanes 7, 9, 10, 11, 15). Ten of the 19 mutants show a loss of the 22 kb fragment and a gain of a new fragment of ~33 kb (pattern B, lanes 1-6, 8, 13, 14, 16). This change in the fragment containing exons 2 and 3 was confirmed by use of Hind III which showed a loss of the 7 kb exon 2+3 fragment and a gain of an 11 kb fragment. Three mutants showed only loss of the 22 kb fragment (pattern C, lanes 12, 17, 19) and 1 mutant showed loss of all 3 fragments (pattern D, lane 18). In B), the TcR β gene rearrangement patterns for these 19 mutant colonies is shown. The 10 mutants which showed the *hprt* exons 2-3 molecular weight (MW) change have identical TcR β patterns (pattern 6, lanes 1-6, 8, 13, 14, 16). The 3 mutants which demonstrate the loss of the exons 2-3 fragment share a different TcR β pattern (pattern 11, lanes 12, 17, 19). The exons 2-9 deletion mutant has another unique TcR β pattern (pattern 12, lane 18). Lastly, the 5 mutants with no *hprt* change show 2 patterns different from the others with 4 mutants sharing the same pattern (pattern 1, lanes 7, 10, 11, 15) and a single mutant with a unique pattern (pattern 2, lane 9). Thus these 19 mutants represent 5 different mutations by TcR gene rearrangement analysis.

Our results with the initial *in vitro* assay demonstrated that most of the mutations obtained did not represent independent mutations (as seen in Figure 1). Thus, there is over growth of some mutants during phenotypic expression. To resolve this, we have developed an *in situ* method for the *in vitro* assay. Briefly, on day 2 cells are innoculated at 5 x 10^3 cells/well in growth medium with 0.25μg/ml PHA and 1 x 10^4 feeder cells in the absence of TG. Three days are allowed for growth, after which the cells receive 50μl of 75% IL-2, 20% HL-1, 5% serum, 0.25μg/ml PHA, 2 x 10^5 feeder cells and 50μM TG. Plates are read and mutants isolated after 10-14 days. Results of recent *in vitro* assays using both the mass culture and *in situ* techniques are shown in Table 1. Using the *in situ* method, most mutants represent independent mutations. This method is now used routinely for *in vitro* experiments.

TABLE 1 - *hprt* mutations induced *in vitro* by gamma irradiation (300 rads)

Expt.	#wt	#mutants	hprt alterations	TcR sets
LS345 (in situ method)	3	35	3 exon 1-9 deletion	none
			1 exon 3 deletion	
			1 exon 2 deletion	
			1 exon 4 deletion?	
			1 exon 1+2 or 2+3 deletion	
			2 loss exon 5-9 + 2 new	
			2 loss exon 5-9 + new	
			1 loss exon 2 + new	
			1 change exon 1	
			1 change exon 6	
			21 no change	
LS450 (in situ method)	5	30	3 exon 1-9 deletion	
			1 exon 2-9 deletion	
			1 exon 1 deletion	
			2 exon 4 deletion?	doublet
			1 exon 2+3 deletion	
			1 exon 2+3 deletion (VDJ)	
			4 loss exon 5-9 + new	
			1 loss exon 3 + new	
			2 exon 2 deletion?	
			1 exon 1-3 deletion (+new?)	
			1 loss exon 2 + new	
			1 loss exons 1-4 + new	
			1 loss exon 4 + new	
			8 no change	doublet, 1 ND
LS450 (mass culture method)	0	18	2 exon 1-9 deletion	
			13 loss exon 3 + new	13-mer
			1 loss exon 4 + new	
			2 no change	

2) Studies of radiation effects of ^{201}Thallium using the *in vitro* Clonal Assay

During year 2 of the current funding interval, we performed a study of the *in vitro* mutagenicity of Auger electrons and other low energy irradiations emitted by ^{201}Thallium. This was to determine the effects of such low level radiations on lymphocytes. Although Auger electrons have a very short range, thallium is concentrated in cells as a K^+ analog. Briefly, cells were exposed to ^{201}Thallium *in vitro* and the mutant frequency measured by the methods developed in the study of gamma irradiation.

For the 20 hour treatment, ^{201}Thallium was added to a final concentration of 5μCi/ml to one cell culture. These cells were then incubated for 20 hrs, at 37°C (5% CO_2/94% air). One hour prior to the end of the incubation, one cell culture was irradiated with 300 rads external beam

gamma radiation (^{137}Cs irradiator), and another was treated with ^{201}Thallium at a final concentration of 100μCi/ml for 1 hour. The cell cultures were then washed five times to remove essentially all of the residual thallium, primed with 1μg/ml PHA and incubated for 40 hrs. Cells were then plated for cell survival. Cells were subcultured for 72hr and then subcultured again. Finally, cells were plated for an *in vitro* assay and scored at 14 days.

We first determined the dose response to *in vitro* exposure to ^{201}Thallium. T-lymphocytes were treated as described but at ^{201}Thallium concentrations of 2.5, 5.0, and 7.5μCi/ml for 20 hours. These results indicate that mutant frequency increases with thallium dose (Figure 2). The data were compared to 300 rad external beam gamma radiation controls. 5.0μCi/ml ^{201}Thallium was determined to give equivalent mutant frequencies and chosen for use in future studies.

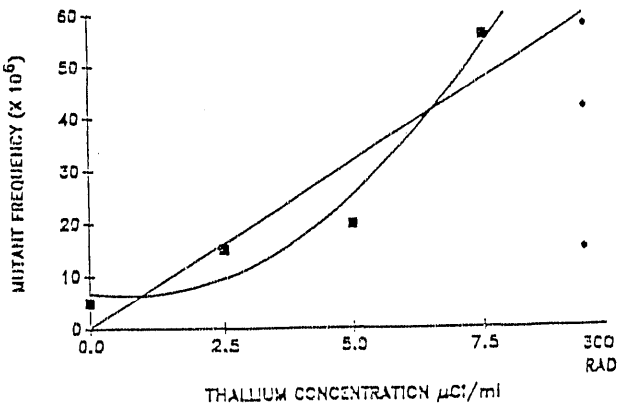


FIGURE 2 - Mutant frequency after *in vitro* exposure to ^{201}Thallium

We then determined the effect of ^{201}thallium dose rate on mutant frequency. We treated T-lymphocytes as described with 300 rads external beam gamma radiation, 5μCi/ml ^{201}Thallium for 20 hours, and 100μCi/ml ^{201}thallium for 1 hour and assayed for cell survival and induced mutant frequency. The survival data for two individuals show average relative survival of 9.1% following treatment with 300 rads; 7.2% following 5μCi/ml ^{201}Thallium, 20hrs; and 0.43% following 100μCi/ml ^{201}Thallium, 1hr. The induced mutant frequency was then determined in one individual. Background *in vivo* mutant frequency was 6.7 X 10^{-6}. Induced mutant frequencies following 300 rad gamma, 5μCi/ml ^{201}Thallium, and 100μCi/ml ^{201}Thallium were 17 X 10^{-6}, 28 X 10^{-6}, and 180 X 10^{-6} respectively.

In summary, 5μCi/ml ^{201}Thallium closely approximates (both in cell survival and in induced mutant frequency) that of 300 rads external beam gamma radiation. Treatment with an equivalent dose of thallium but at a much higher dose rate (100μCi/ml, 1hr) reduces cell survival 17 fold relative to 5μCi/ml ^{201}Thallium. Concomitantly, the higher dose rate results in a greater than 6 fold increase in mutant frequency.

3) Demonstration of Ara C Enhancement of Radiation Effects in the *In Vitro* Clonal Assay

An increase in the frequency of X-ray induced chromosome aberrations by incubation with cytosine arabinoside (ara-C) has been reported with G_0 phase human lymphocytes (Preston, 1980). This increase was proposed to be the result of the accumulation of regions of repair of the radiation induced damage which, upon release of the ara-C inhibition by deoxycytidine (dC), can interact to yield chromosome interchanges. To investigate the extent of radiation damage repair, we initiated studies employing incubation with ara-C to inhibit repair of this damage, followed by release in the presence of dC to allow completion of the repair of the accumulated repairing regions. Interaction of repairing regions, which results in an increase in chromosome aberration frequency, might also result in an increase in mutation frequency (Preston, 1980). In addition, those induced mutations should primarily result from deletion/interchange events which should be detected by Southern blotting studies.

Cultures were exposed to 200 cGy gamma irradiation and incubated with 50 uM ara-C for 1, 2 or 4 hours post treatment. Unirradiated cultures were also incubated with ara-C. After the ara-C incubation, the cultures were washed and put into medium containing 100 uM dC. The cultures were then incubated with PHA for 36-40 hours to achieve mitogen stimulation, and plated for cloning efficiency determinations as described in above. Aliquots of these treated cultures were also subcultured for expression of the TG^r phenotype after mitogen activation with PHA (day 2) and at 3 day intervals thereafter. These cultures were plated to determine the mutant frequency on day 8, a time found optimal for the expression of mutants induced by gamma irradiation alone. The results are summarized in Table 2 for mutation induction by irradiation plus ara-C treatment. Exposure to either 200 cGy of radiation alone, or to ara-C alone results in a slight increase in mutant frequency. However, incubation with ara-C after irradiation results in a large increase.

TABLE 2: Induction of Mutation by Irradiation plus ara-C[a]

	Mutant Frequency x 10^{-6} [d]			
Exp. No.	0 cGy (A)	0 cGy + ara-C (B)	200 cGy (C)	200 cGy + Ara-C (D)
LS148	3.5	10.9	9.2	232.1
LS160	2.8	25.1	16.4	141.6
LS267	1.9	2.4	17.1	101.2
LS273	1.6	4.7	10.9[b]	64.6[b]
LS359	8.0	17.7	24.7[c]	91.9[c]
Mean ± SD	3.6 ± 2.3	12.2 ± 8.4	15.7 ± 5.5	126 ± 58.4[e]

[a] Cells were irradiated (0 or 200 cGy), incubated with 0 or 50 μM ara-C for 4 hrs and mutant frequency determined on day 8 of subculture expression
[b] In situ expression: 9.6 x 10^{-6} (200 cGy) and 46.8 x 10^{-6} (200 cGy + ara-C)
[c] In situ expression: 18.7 x 10^{-6} (200 cGy) and 101.9 x 10^{-6} (200 cGy + ara-C)
[d] Significantly different ($p < 0.05$) from the 0 cGy value.
[e] Significantly different ($p < 0.05$) from the 200 cGy value.
[d] Statistical analysis: Significant differences ($p < 0.05$) were found in comparisons of A vs. D, B vs. D, and C vs. D. Non-significant differences were found in A vs. B, and A vs. C.

If the induction of mutations is due to chromosome interchange events which involve the *hprt* gene, large structural alterations or deletions of this gene should predominate. These types of mutations represent 10-15% of the mutations occurring *in vivo* in unirradiated individuals (Nicklas et al., 1987; 1989), but increased to 33% in a group of individuals exposed *in vivo* to ionizing irradiation (Nicklas et al., 1990). *In vitro* exposure to 300 cGy gamma irradiation

results in 75% of mutations showing structural alterations of the hprt gene (see above and O'Neill et al., 1991b). To further characterize the mutations induced by irradiation plus ara-C treatment, Southern blot studies were performed. A total of 40 mutant colonies were isolated. Analysis of the TcR gene rearrangement patterns revealed that these represented 8 independent mutations of which 4 (50%) had alterations.

The recovery of TcR/*hprt* siblings in these studies results in an inefficient analysis of mutation events. However, use of the *in situ* assay described above should result in the isolation of a higher proportion of mutants resulting from independent mutations. In cultures exposed to 200 cGy plus ara-C, day 5 in situ expression yielded mutant frequencies of 46.8 and 101.9 x 10^{-6}, compared to 64.6 and 91.9 x 10^{-6} with day 8 subculture expression. Nineteen mutant colonies were isolated and analyzed by Southern blotting for TcR pattern and *hprt* alterations. By TcR gene rearrangement patterns, the 19 mutants represented 17 independent mutations. Ten (59%) of these showed *hprt* alterations including simple deletions (5) and loss of fragments with the gain of new fragments (MW change, 5). Thus in the total of 25 mutations, 14 (56%) showed *hprt* alterations. Since 200 cGy alone causes few mutations, our interpretation of our results is that the amount of damage induced by only 200 cGy of irradiation is repaired over a time interval which results in minimal interaction of repairing regions. Either the accumulation of repairing regions with ara-C or the induction of more DNA damage to be repaired as with exposure to 300 cGy results in an increased probability of the interaction of repairing regions. These interactions yield mutations which result from large structural alterations of the *hprt* gene.

4) Molecular Mapping of the *hprt* Region of Xq26 and Placement of Mutant Deletion Breakpoints

Because a large number of anonymous probes map to the Xq26 region near the *hprt* gene, we were able to determine if any of these markers were co-deleted in our deletion mutants. In an initial study (Nicklas et al, 1991a), a total of 57 *hprt* mutant T-cell clones arising *in vivo* in normal young adults, elderly adults and newborns and in irradiation or cis-platinum exposed individuals were studied as well as mutants from *in vitro* irradiated T-lymphocyte cultures. These particular mutant clones were chosen because they had deletions of *hprt* which extended either 5′ (e.g. an exon 1 deletion) (11 mutants), 3′ (e.g. an exon 4-9 deletion) (10 mutants) or 5′ and 3′ (i.e. total *hprt* gene deletions) (36 mutants) of the *hprt* gene. Twenty anonymous probes were tested for co-deletion with the *hprt* gene by Southern blotting methods (DXS10, DXS11, DXS19, DXS37, DXS42, DXS51, DXS53, DXS59, DXS79, DXS86, DXS92, DXS99, DXS100d, DXS102, DXS107, DXS144, DXS172, DXS174, DXS177, DNF1). Five of these probes (DXS10, DXS53, DXS79, DXS86, DXS177) showed co-deletion with *hprt* in some mutants. The mutants established the following unambiguous ordering of the probes relative to the *hprt* gene and each other: centromere - DXS53 - DXS79 - $^{5'}$*hprt*$^{3'}$ - DXS86 - DXS10 - DXS177 - telomere. Since that report, we have mapped an additional probe, DXS311 (courtesy of J.J. Holden), between *hprt* and DXSX86 and have studied an additional 90 mutants. The most current map is shown in Figure 3 with the number of breakpoints mapped between each probe shown above.

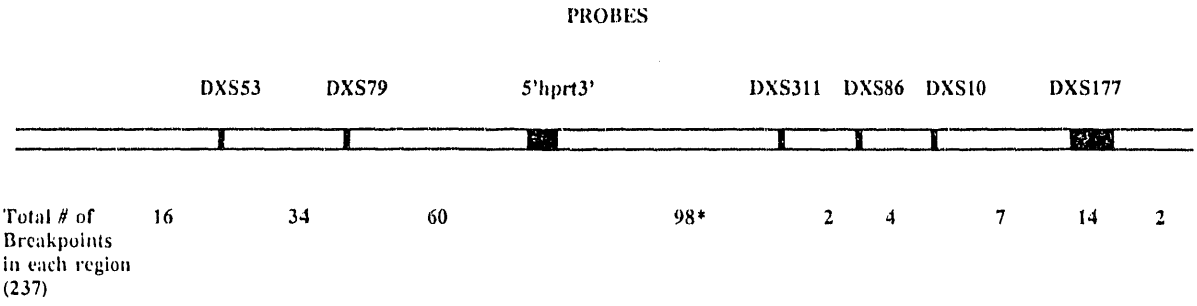


FIGURE 3 - Mapping of anonymous DNA sequences near the *hprt* gene

Pulsed field gel electrophoresis (PFGE) is being used to restriction map the *hprt* region of Xq26 with rare cutter enzymes and the linked probes. Average fragment sizes for 5 rare cutter restriction enzymes are listed in Table 3. Figure 4 shows the derived rare-cutter restriction map of the *hprt* region. Assuming that no gaps exist in the map, the region from DXS53 to DXS177 spans about 2Mb. Therefore deletions of at least this size can be tolerated at *hprt* as several mutants have deleted all markers from DXS53 to DXS177. Our map distance for the DXS79-DXS177 region is supported by the recent report of Dr. Schlessinger's group at Washington University who have YAC mapped 8Mb in Xq26 (Little et al., 1992). However, their contig map does not include DXS53. [It maps off the centromeric edge of the contig (our results and G. Pilia, personal communication).] Dr. Schlessinger has made a set of YACs spanning this region available to us including some containing DXS53.

TABLE 3 - Pulsed field restriction fragments in kb for *hprt* and linked probes

		PROBE						
ENZYME	EXPT.	DXS53	DXS79	hprt	DXS311	DXS86	DXS10	DXS177
KspI	AVE(3).	515 (764)[1]	245 (777)	245 (378)	384	384	111	510,(824) (731),(563)
SfiI	AVE(2).	335 (497)	442	192	326	326 (<50)	123 (525)	206, 520 (628)
BssHII	AVE(2).	517	254,(396) (738)	125	476	476	476	512 298
FspI	AVE(3).	528, (94) (375)	450 (590)	234, 502 (607)	388	388 (470)	114	507, 337 246,(400)
NotI	AVE(2).	>1Mb	>1Mb	>1Mb	379	379	83	466

[1] numbers in parenthesis indicate fragments seen inconsistently or very faintly on Southern blots.

Mapping of deletion endpoints in *hprt* mutants is currently underway. A reasonably precise estimation of the size of a deleted region can be determined based on size changes of the restriction fragments, i.e. those containing non-deleted anonymous sequence being probed. Initial studies with 3 mutants indicates that one (MF52 A10H6) has a very small deletion of exon 1 and 5' *hprt* untranslated sequence, a second (MF33 A4G5) has a telomeric deletion spanning ~1200kb and a third has a telomeric deletion of >1200kb. Figure 5 shows a DXS177 probed pulsed field blot of several mutant and wild type DNAs.

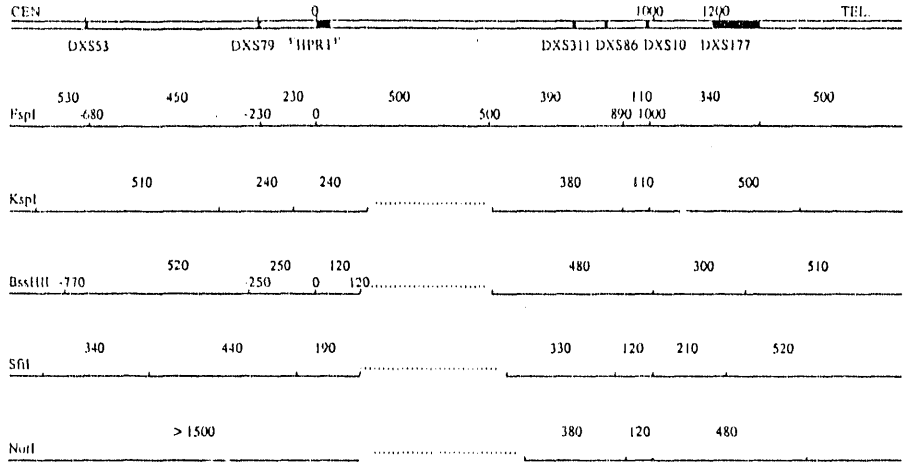


FIGURE 4 - Pulsed field map of the *hprt* gene region. Black boxes indicate probe sequences mapping to the *hprt* region of Xq26. Horizontal lines below the double line of the *hprt* region are restriction maps for 5 rare cutter restriction enzymes. Numbers directly above vertical lines indicate distance in Kb from *hprt*. (*Hprt* being 0, distance centromeric is listed as negative while distance telomeric is positive) Dotted lines indicate regions with certain enzymes for which we do not have contiguous restriction fragments.

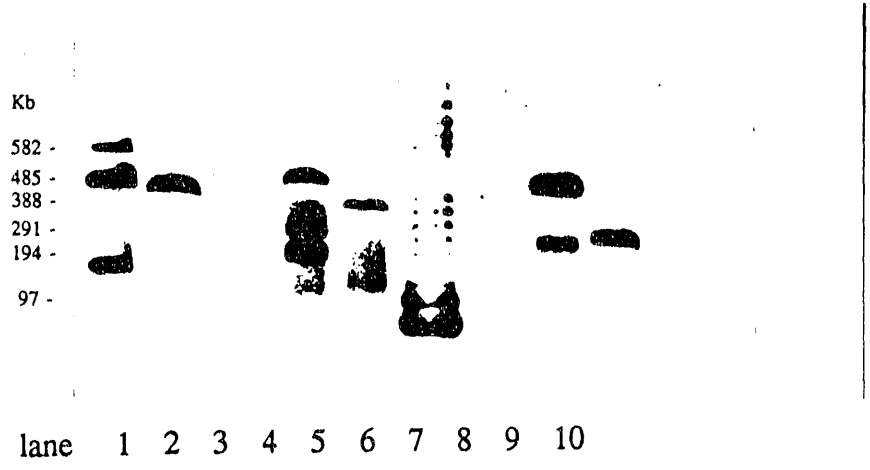


FIGURE 5 - Wild type and *hprt* mutant T-cell DNA's were restricted with SfiI (lanes 1-3), FspI (lanes 4, 5, and 7), and BssHII (lanes 8-10) and separated with the BioRad CHEFII PFGE system (165V, pulse times of 80s for 16h, 100s for 5h, and 120s for 6h) and probed with DXS177. Lanes 1, 4 and 8 are wild type PBL DNA. Mutant MF33 A4G5 (lanes 2, 5, 9) is a mutant which has lost 3' *hprt* sequence to part of DXS177. Mutant LS252 A10F9 (lanes 3, 7, 10) is a mutant which has deleted 3' *hprt* sequence to all of DXS177. Lane 6 contains lambda concatemers.

Over the course of our studies of over 1600 mutants by Southern blotting, we have collected

~400 mutations which have visible alterations in their *hprt* gene. Mutants come from a variety of treated and non-treated individuals (normal young adults, normal aged adults, cis-platinum treated patients, fathers of Prader-Willi children, RIT patients, newborns) as well as *in vitro* radiation studies. The mutants have been divided into those with definite deletions; single breakpoint mutants (possible translocations - fragment size change); VDJ recombinase mutants; and other (complex or ill defined). Some placements are subjective (e.g. exon 7-9 region loss with new fragment) and require further molecular study.

Figure 6 shows the breakpoints of the simple deletion and single breakpoint mutants with definitely placed breakpoints (the 38 exon 2+3 VDJ recombinase mutants are excluded). Using the summary information on mutants in Figure 6, one can search for hotspots of mutation by determining the distribution of definitively placed breakpoints over the introns in relationship to intron size. The distribution of breakpoints does not fit a χ^2 distribution based on relative intron size ($p=0.0006$). There are too many breaks in intron 3 and too few in intron 6. These results are suggestive but the fact that many of the breakpoints are excluded from the analysis as they are not definitively located could skew the results (e. g. breakpoints in the exon 7-9 region). Further studies (PCR) will be needed to map these breakpoints. Of note in Figure 6 is that there are a large number (13) of exon 4 deletion mutants. Large numbers of partial and total exon 4 deletions have also been seen by another group (J. Fuscoe, personal communication). Sequencing of several of their partial exon 4 deletions has indicated that they all start at the same position in an AT rich region (a possible topo II site). Among the single break mutants, there are a number of mutants with breakpoints near exon 6 which is also of interest.

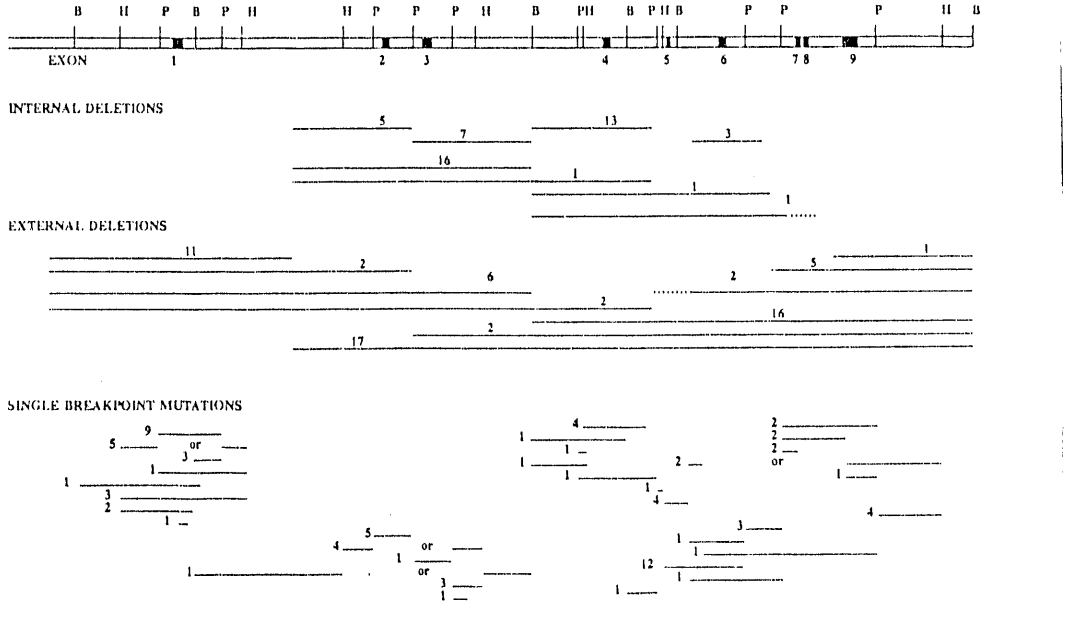
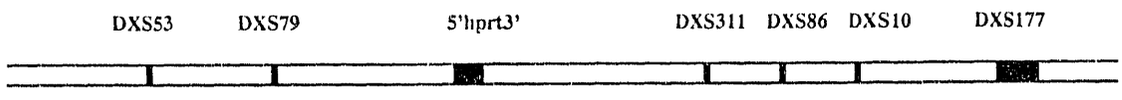


FIGURE 6 - The # and distribution of mutation breakpoints across the *hprt* gene. For the internal and external deletions, the line indicates deletion extent. Endpoints are approximate and only indicate intron placement. For the single breakpoint mutants, the line(s) indicate the range in which the breakpoint lies.

One can also analyze differences in extent of deletion between the different exposed groups, to determine if, for example, mutants exposed to radiation have larger deletions than unexposed mutants. For total deletions (a definitive class), 7/15 adult mutants (47%) have no deletion of linked markers and those that do delete markers are evenly split between deleting 5' (DXS79), 3' (DXS311) or both. In contrast, in RIT patients, only 7/28 (25%) of mutants have lost no linked markers and the distribution of breakpoints is skewed 5' (12-5', 3-3' and 6-both). This is even more extreme for *in vitro* mutants (radiation induced) where 0/8 have no deletion of linked markers and the skewing is extreme (5-5', 0-3', 3-both). This indicates that radiation mutants are larger and skewed 5'. Figure 7 shows percentages of mutant breakpoints in each region in normals, RIT and *in vitro* mutants. As can be seen, while the percentages of mutants with different 3' breakpoints are similar in all 3 groups, the irradiation mutants extend more often and further 5'. For example only 5% of normal mutants delete DXS53 but 14% of *in vitro* mutants do. Thus, it will be of interest to sequence these breakpoints to determine the differences in spontaneous and radiation induced mutant breakpoints. Current work in this area includes development of probes for the large region between *hprt* and DXS311, mapping more mutant breakpoints and sequencing mutant breakpoints at the possible "hotspots".



Group (# mutants)										
Normals (56)	5%	7%	25%	(5'-38%, 3'-62%)	46%	0%	2%	4%	11%	0%
post-RIT (88)	7%	19%	22%	(5'-48%, 3'-52%)	41%	1%*	3%	1%	5%	1%
In Vitro (35) (3Gy)	14%	29%	14%	(5'-57%, 3'-43%)	29%	3%	0%	9%	3%	0%

Some mutants were not probed with DXS311 thus the # breakpoints between DXS311 and DXS86 maybe a slight underestimate
*one partial DXS311 deletion

FIGURE 7 - Distribution of Mutant Breakpoints Among Normals, RIT and *In Vitro* Mutants

5) Studies on Radioimmunotherapy (RIT) Patients

Over the past several years, we have performed 2 studies on patients who have received ^{131}I radioimmunotherapy for treatment of hepatoma or cholangiocarcinoma. These individuals receive anti-ferritin antibodies conjugated to ^{131}I. Treatment is usually several doses of 30-50mCi of ^{131}I at about monthly intervals. The first study reported a number of individuals studied mostly 2 months after a treatment. Many individuals had had numerous rounds of treatment. The second study included a number of pre-treatment and up to 3 years post-treatment samples. The results of these studies have been published (Nicklas et al., 1990, 1991b; O'Neill et al., 1992). The main points of the papers are that Mfs are greatly increased after treatment even long term after treatment (mean $27.8(\pm 16.2)\times10^{-6}$ in post-patients versus $11.5(\pm 5.1)\times10^{-6}$ in pre-

treatment patients, $p<0.007$), that although there is a poor correlation of total activity of RIT with Mf there is a good correlation of last activity with Mf (Figure 8). This indicates that the last exposure has the most effect on mutant frequencies. Molecular studies on the mutants from RIT patients show a large increase in % of mutants with structural alterations in the exposed individuals as compared to pre-treatment (Table 4). This is similar to results seen *in vitro* (Table 4). As can be seen in the table, the spectrum of mutation is also very different from normals in that there is a much higher proportion of total *hprt* gene deletions. Although there was no correlation of Mf with total activity (as stated above), there is a good correlation of % of mutants with alterations with total administered activity (Figure 9).

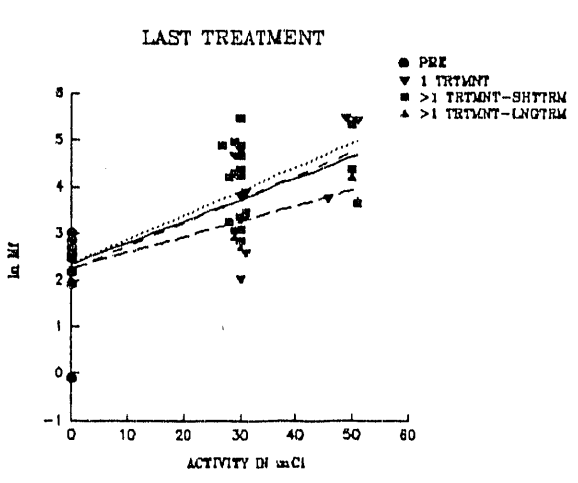


FIGURE 8 - Graph of ln mutant frequency against activity of ^{125}I received in last treatment

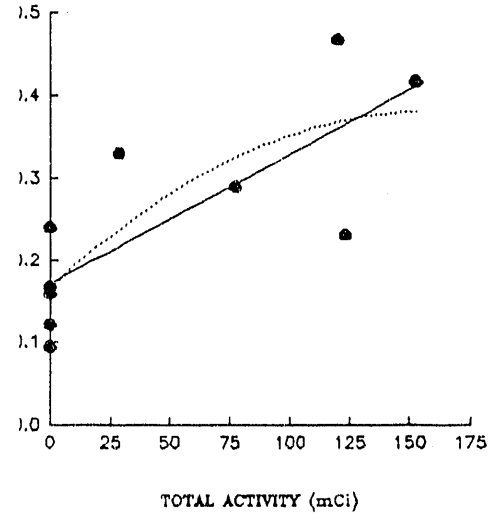


FIGURE 9 - Graph of % of mutations with *hprt* alterations on Southern blot versus total activity of ^{125}I received. Linear (-) and second order (.) regression lines.

TABLE 4 - Spectrum of Normal and Post-Irradiation *hprt* mutations

Group	#Mutations Studied	# with alteration	% with alteration	Total deletion	Partial deletion	Single Breakpoint	VDJ recombinase	Other
Young Adults	492	64	13.0	12	18	18	8 (1.6%)	8
Aged Adults	127	15	11.8	3	8	3	1	0
pre-RIT	118	20	16.9	3	10	6	0	1
post-RIT	235	90	38.3	37	24	22	3 (1.2%)	4
In Vitro (3Gy)	143	66	46.2	12	24	29	0	1
In Vitro (2Gy+ ara-C)	25	14	56.0	2	4	7	0	1

A number of mutants from the RIT patients have been studied by sequencing of PCR products of *hprt* cDNA (Table 5). This work has been performed by Adonis Skandalis, a graduate student in Dr. Barry Glickman's lab as a collaborative effort between our laboratories. Of note in the results is that 6 of 13 mutants (46%) have one entire exon missing from their cDNA presumably due to a point mutation affecting a splice junction. This is a high percentage as compared to normals where our group (Recio et al., 1990) and Dr. Tates' group (Rossi et al., 1990) found frequencies of 25% (8/32) and 23% (7/31), respectively. Further studies will confirm whether this is a real difference in spectra.

TABLE 5 - Sequencing Studies on Mutants from RIT Patients

Experiment	Mutant	*hprt* Change	Alteration
MF123	914	$A_{602}\rightarrow T$	Asp→Val
	902	$T_{596}\rightarrow A$	Val→Asp
	912	- C_{545}	frameshift
	916	- exon 8	splice
	915	- exon 7	splice
	913	- exon 6	splice
MF121A	905	- exon 4	splice
MF126C	924	$A_{586}\rightarrow G$	Asn→Asp
	926	$G_{46}\rightarrow A$	Gly→Ser
	925	+ A	frameshift
	918	- exon 4	splice
MF73	927	Del $bp_{243\text{-}265}$	Del + frameshift
	928	- exon 4	splice

6) Mutant Frequencies of and Molecular Analysis of Mutants from Plutonium Workers

Although, these individuals were not listed as an exposed group to be studied on previous proposals, we recently had the opportunity to obtain blood samples from 17 individuals who handled plutonium for the Manhattan project. These individuals have been extensively studied (Voelz and Lawrence, 1991) and urinary measurements of plutonium made. Table 6 lists information on the workers and their determined mutant frequencies.

TABLE 6 - Summary of Information on Plutonium Workers

EXPT.	ID*	AGE	CE	Mf	BQ (1987)	TOTAL REC'D GBq-s	CUMULATIVE RED MARROW DOSE mSv	CUMULATIVE WHOLE BODY DOSE mSv	SMOKING STATUS
LS534J	---	49	0.06	44.0	0	0	0	0	ND
LS534K	---	36	0.11	6.3	0	0	0	0	ND
LS535I	---	44	0.22	16.9	0	0	0	0	ND
LS535J	---	36	0.12	23.5	0	0	0	0	ND
LS534D	13	74	0.11	75.7	52	65	55.2	92.6	EX
LS535C	26	68	0.24	20.8	110	148	125.1	210.1	EX
LS534E	2	70	0.10	50.4	270	379	320.1	537.6	EX?
LS534A	22	68	0.15	39.3	290	230	194.0	325.6	EX
LS535B	?	69	0.18	29.7	296	(235)	(197.0)	(330.8)	ND
LS535E	23	66	0.28	27.0	310	556	468.9	787.3	NON
LS535D	21	66	0.58	12.1	370	514	433.4	727.8	EX
LS534C	19	67	0.11	38.2	410	648	638.3	918.0	NON
LS535A	20	69	0.16	37.6	480	882	743.7	1248.8	CUR
LS534F	12	80	0.14	62.2	560	1020	862.9	1448.9	NON
LS535G	17	69	0.29	23.0	1260	2250	1901.1	3192.2	EX
LS534B	1	73	0.07	46.6	1590	2460	2423.1	3490.0	EX?
LS535H	5	73	0.30	23.7	1630	3670	3092.9	5193.6	NON
LS534G	8	68	0.08	26.3	1780	1280	1819.4	1083.5	NON
LS535F	9	76	0.22	8.4	2000	3540	2984.6	5011.6	EX
LS534H	4	71	0.12	5.8	2150	3810	3211.1	5392.0	CUR
LS534I	7	83	0.11	89.5	3180	2710	2285.2	3837.3	NON

* Voelz and Lawrence, Health Physics 61:181 (1991)

Although it was difficult to ascertain a dose effect based on Voelz and Lawrence's estimated exposure (Figure 10), the mutant frequencies for this group were significantly elevated as compared to age matched controls (39.1×10^{-6} versus 14.6×10^{-6}, $p < 0.0005$).

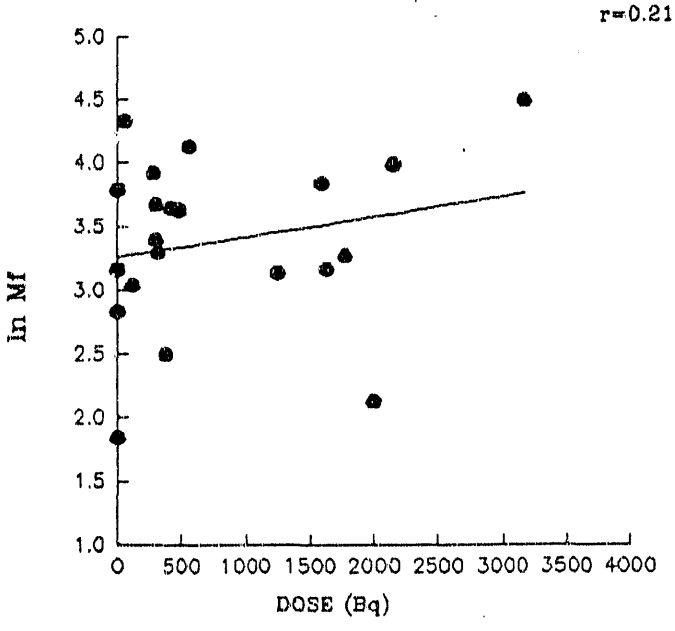


FIGURE 10 - ln Mf versus Dose for Plutonium Workers

In addition, when the Mf values for the exposed workers were placed on the regression line for normals (Figure 11) they could be seen to be elevated as compared to normals. An additional factor (0.53) was needed to bring these individuals onto the regression line (Figure 12).

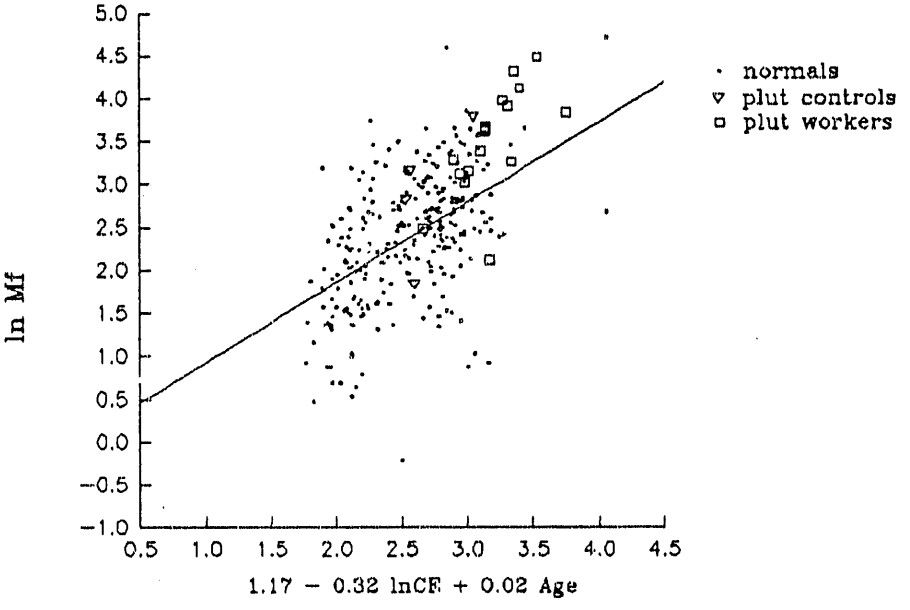


FIGURE 11 - Plot of ln Mf versus expected Mf for normals and plutonium workers

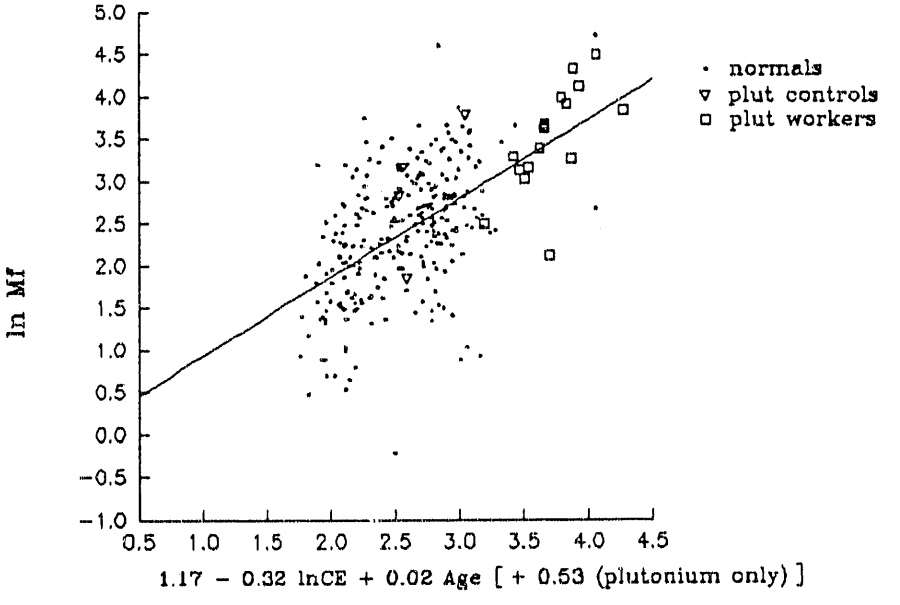


FIGURE 12 - Plot of ln Mf versus expected Mf with correction factor for plutonium workers

Molecular analysis of 120 mutants from 17 individuals was performed (Table 7). Expectation

was that a large fraction of the mutations would have changes on Southern blots, like RIT mutants; however, only 13% of plutonium workers' mutations had changes. This compares to 157 mutations from 12 aged matched controls where 11% of the mutations had changes.

TABLE 7 - Southern Blot Results on Mutants from Plutonium Workers

EXPT.	Mf ($x10^{-6}$)	#OF CLONES WT	#OF CLONES M	*hprt* gene changes	TcR Sets
LS534H	5.8	3	8	8 no change	
LS535F	8.4	1	1	1 no change	
LS535D	12.1	1	6	(1) -2+3,-4,-5-9 (1?) 7 no change	
LS535C	20.8	0	8	1 total deletion 7 no change	
LS535G	23.0	3	8	3 Δ exon 7-9 5 no change	triplet
LS535H	23.7	3	20	1 Break in exon 2+3 2 Δ exon 4-9 17 no change	 doublet
LS535G	26.3	2	2	2 no change	
LS535E	27.0	1	6	6 no change	doublet
LS535B	29.7	2	11	2 total deletions (1) break in exon 2+3 (1) Δ exon 1 (1) Δ exon 6 + new frag. (6) no change	
LS535A	37.6	3	6	6 no change	
LS534C	38.2	4	4	4 no change	doublet
LS534A	39.3	2	12	1 total deletion 11 no change	
LS534B	46.6	1	1	(1) Δ 4-9 + new frag.	
LS534E	50.4	0	9	(1) Δ 1-3 + new frag. 8 no change	 doublet
LS534F	62.2	0	2	2 no change	
LS534D	75.5	4	14	14 no change	
LS534I	89.5	2	2	(1) Δ exon 1	
TOTAL		32	120	17/120 (14%) mutants 15/114 (13%) mutations	114/120 (95%)

7) Study of Airline Pilots

Because airline pilots and flight attendants are exposed to radiation, we initiated a study of 21 current and retired airline pilots. This study was performed in conjunction with Dr. James E. Crane who has access to a large number of pilots. Table 8 shows the data on the pilots including mutant frequency.

TABLE 8 - Summary of data on airline pilots and flight attendants

SAMPLE #	SEX	AGE	CE	MFx10^{-6}	ACT/RET	HI/LOW ALT
1	M	60	0.34	16.2	R	LO
2	M	61	0.26	35.1	R	HI
3	M	62	0.66	11.5	R	HI
4	M	46	0.62	6.1	A	LO
5	M	62	0.31	9.8	-	-
6	M	62	0.25	6.3	A	LO
7	M	59	0.63	13.7	A	HI
8	M	60	0 44	14.7	R	HI
9	F	58	0.81	7.5	A	HI
10	M	49	0.45	8.4	A	?
11	M	65	0.41	7.1	R	HI
12	M	53	0.44	6.9	A	?
13	M	53	0.22	6.7	A	LO
14	M	76	0.45	13.1	R	HI
15	M	57	0.65	8.5	R	HI
16	M	44	1.14	5.9	R	HI
17	M	64	0.13	21.5	R	HI
18	M	62	0.64	12.6	A	HI
19	M	77	0.31	22.2	A	HI
20	F	51	0.64	15.3	A	?
21	M	53	0.49	13	A	HI (?)

Figure 13 shows the Mf of the pilots plotted versus estimated flight time. There is a slight increase in Mf with number of hours but this is not significant.

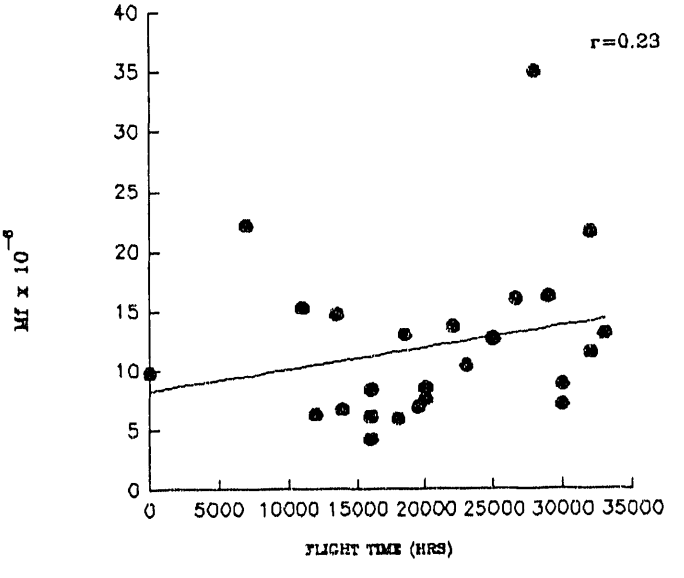
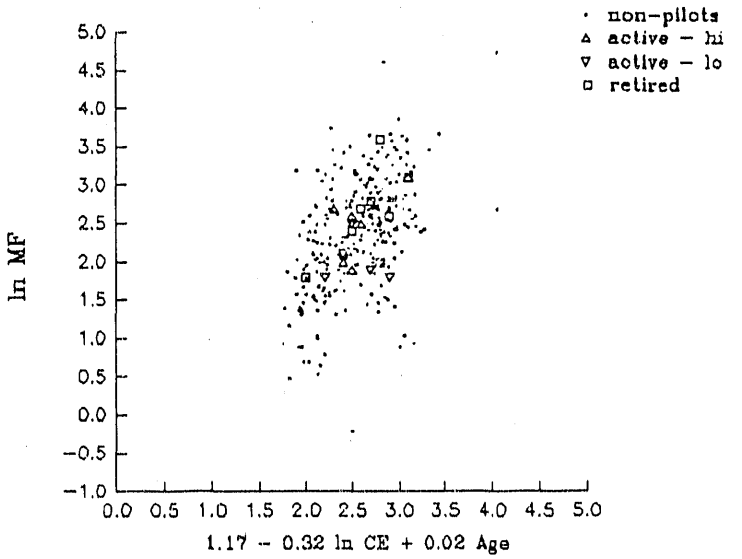


Figure 14 shows the ln Mf of the pilots plotted versus an expected ln Mf based on the empirically derived relationships of age and CE overlaid on the Mf of normals versus expected Mf. The pilots appear to fall within the normal range.



8) Studies of Individuals Exposed in the Chernobyl Accident

We have had the opportunity to obtain 4 samples from a family that immigrated from Kiev after the Chernobyl accident. These samples were obtained in January 1991. Table 9 below gives the sample information.

TABLE 9 - Information on individuals exposed from Chernobyl accident

Expt.	Sex	Age	CE	Mf	ln Mf	Expected ln Mf*
LS460B	F	35	0.25	10.1	2.31	2.31
LS450A	M	35	0.16	7.9	2.06	2.45
MF451D	M	11	0.29	4.3	1.45	1.78
MF451C	F	63	0.19	27.6	3.32	2.96

*1.17 - 0.32 x lnCE + 0.02 x age (in years). [This equation is based on the empiric relationships of lnMf with lnCE and lnMf with age and gives an "expected Mf" based entirely on the CE and age values. Any increase in Mf over this expected value is assumed to be due to exposure to the agent of interest, e.g. radiation exposure. We are currently preparing a manuscript on these values for normals (Branda et al., in preparation).]

Since we have no pre-exposure data on these individuals, it is difficult to know if these values are increased. However, only the ln Mf value for the grandmother (MF451C) is greater than the expected value for a normal of that age with that CE (last column). This suggests that these individuals did not receive a high exposure. Molecular analysis has been performed on the mutants from the 2 males (Table 10). The % of mutations with alterations is 12.5% and 14.3%, respectively which are both normal values.

TABLE 10 - Molecular alterations in *hprt* mutants from Kiev residents

Expt.	#wt	#mutants	*hprt* alterations	TcR sets
LS460A	2 (same TcR)	8	1 exon 1-9 deletion 7 no change	
MF451D	1	7	1 loss exon 6 + new 6 no change	 doublet

9) PCR and Sequencing of *hprt* Mutants

Our laboratory has sequenced the *hprt* cDNA of a number of *hprt* mutant clones from normals in collaboration with Drs. Les Recio and Thomas Skopek (Recio et al., 1990). These mutants showed a variety of mutations including transitions, transversions, frameshifts, small deletions and deletions of whole exons (probable splice site mutations). We have recently begun sequencing analysis of mutants in our laboratory. Briefly, the method (Yang et al., 1990) involves NP40 treatment of about 1000 mutant cells, production of a cDNA with M-MuLV reverse transciptase, PCR with 2 sets of nested primers and then direct sequencing of the purified product using three primers. Figure 15 shows the partial sequence of an *hprt* mutant.

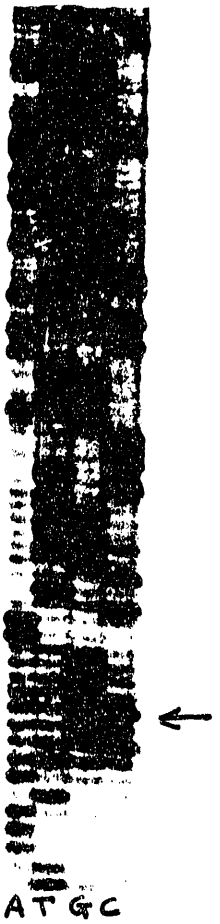


FIGURE 15 - *hprt* sequencing gel using a primer for the 5' part of the *hprt* gene. The sequence

of a mutant (LS530 M356) is shown. Arrow indicates mutation site.

From left to right, the lanes show the bases A, T, G and C. The right-most lane shows that this mutation was a G → C transversion at base No. 207 (exon 3). The normal sequence of AAG GGG GGC has been changed to AAG GGG CGC resulting in the amino acid change of glycine to argenine (amino acid #70). This is the only alteration found in the *hprt* sequence. The mutation is clearly seen as a G→C transversion at position 207 which causes glycine→arginine in the protein sequence.

Multiplex PCR (Gibbs et al., 1990) is being used to define exact exon loss in mutants that have inconclusive patterns on Southern blotting. Multiplex PCR uses 8 pairs of primers (1 pair for each exon except 7+8 which are co-amplified) to PCR 8 fragments from genomic DNA which are easily resolved on an agarose gel. A mutant having 1 or more exons deleted in its genomic DNA will have no corresponding fragment on the gel. An example of a mutant that needs multiplex analysis would be one that shows loss of the PstI exon 7-9 fragment but also a new fragment. This fragment could represent exon 7, exon 9, exon 7+8, etc. Multiplex analysis will resolve which exons are present and which are deleted. Figure 16 shows an ethidium bromide stained gel of products from the multiplex PCR of the *hprt* gene in several mutants.

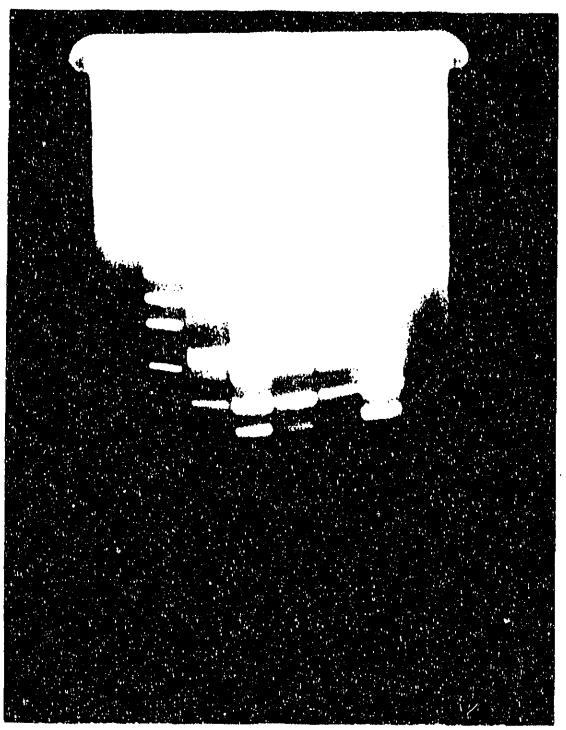


FIGURE 16 - Agarose gel of *hprt* multiplex PCR of mutant and wild type clones.

Lane 1 (left-most lane) contains the molecular weight standards (ϕX174 digested with Hae III). Lane 2 contains the product from an unselected (wild type) clone; lane 3 is a mutant showing

a loss of exons 7-9 on Southern blot studies; lane 4, loss of exons 2 and 3 on Southern blot, lane 5, a total *hprt* deletion on Southern blot; and lane 6, a wild type clone. Lanes 2 and 6, show the normal pattern of exon containing PCR products. They are from top to bottom, exon 7 + 8 (1533 bp), exon 9 (1278 bp), exon 3 (1059 bp), exon 5 (707 bp), exon 1 (626 bp), exon 2 (572 bp), exon 6 (441 bp), and exon 4 (334 bp). The mutant DNA in lane 3 shows the loss of only exon 9. Thus, this mutant must be only an exon 9 deletion. Lane 4 shows the loss of exons 2 and 3 as expected, while the mutant DNA in lane 5 shows all fragments faintly (except for exon 4). It also has a new fragment slightly larger than exon 5. The faint fragments are undoubtedly due to background from the feeder cells (TK6). This cell line has an exon 4 deletion which is why exon 4 is not seen. The larger molecular weight exon 5 product probably reflects PCR across the deletion breakpoint (we are presently analyzing this deletion mutation in the TK6 cells). Because of these results, we are investigating the use of a TK6 derivative which contains a total *hprt* deletion as a feeder cell. We have two cell strains, obtained from Dr. Howard Liber, which contain large deletions involving the *hprt* gene. Strain 36 x 4 is deleted for DXS 79 - *hprt* - DXS 311 - DXS 86 - DXS 10 - DXS 177 and strain 39 x 1 is deleted for DXS 53 - DXS 79 - *hprt* - DXS 311 - DXS 86 - DXS 10. The use of a large deletion feeder cell will remove the potential complication of DNA contamination with both the multiplex PCR and the pulse field gel electrophoresis studies.

10) Development of an HLA Clonal Assay

While the *hprt* assay is well characterized and used worldwide, it is based on only 1 X-linked gene. It is important to develop other assays at other, especially autosomal, genes to confirm the results seen with the *hprt* assay. It is difficult to find other suitable genes because of the need for an easy selection system and the need for selection of heterozygotes. Dr. A. Morley has published several papers on the use of a human HLA (major histocompatibility gene) clonal assay (Janatipour et al., 1988; Turner et al., 1988; McCarron et al., 1982; Morley et al., 1990). These genes reside as a cluster on chromosome 6p and are extremely polymorphic. Antibodies exist which react specifically to the proteins coded for by different alleles. Because of the large number of alleles at each gene, almost every individual is heterozygous and selection using antibodies against one allele is possible to obtain mutants at that allele. Although we have attempted this assay in our laboratory and have obtained some mutants, in our hands, we obtain numerous phenocopies. We have thus chosen several modifications of the assay. We are using different antibodies which are better fixers of complement and a fluorescent cell sorter to select mutants. In particular, we are first using anti-class I Ab to select cells expressing HLA class I molecules and then selecting mutants which do not have a particular allele at the class I HLA-A gene.

Our initial studies to measure mutations at the HLA locus employed anti-A2 antibody and peripheral blood T-lymphocytes. We felt it necessary to develop selection of *in vivo* arising mutants before we initiated studies of *in vitro* induction with gamma irradiation.

Since we are using selection against HLA A2 in these studies, it is necessary to identify individuals who are A2 heterozygotes (e.g. A2/AX, X≠2). We have typed 15 individuals for their HLA A and B alleles using Red Cross basic HLA trays and Pel-Freez complement. The

results are shown in Table 11. Nine of the 15 individuals were definitely A2 heterozygotes with 3 others being probable A2 homozygotes. The frequency of the A2 allele is ~28% in Caucasians which would predict 40% A2 heterozygotes and 8% A2 homozygotes. Thus our frequencies of A2 individuals is somewhat higher than expected.

TABLE 11 - HLA A and B typings of 15 individuals

	HLA A		HLA B	
Individual	allele 1	allele 2	allele 1	allele 2
BC	1	29	8	13
KR*	2	9	7	10
SL*	2	29	13	15
SC	1	29	13	16
AS	3	29	7	12
JS*	2	30	12	w53
JD*	2	3	21	w60
BH*	2	9(24)	7	15
SR	2	-	12	-
MC*	2	11	27	-
CV	2	-	16	-
DJ*	1	2	12	38
ML*	2	3	12	-
AB*	2	3	12(44)	35
PN	2	-	12(44)	17

*Definitely A2 heterozygous individual
-No second reaction, possibly homozygous

We obtained the BB7.2 hybridoma which makes an anti-A2 antibody from the ATCC. These cells were grown in RPMI with 10% serum for 6 days before injecting into Charles River Balb/c mice. Twenty-two mice were pristane primed and irradiated with 500 cGy of gamma irradiation. Approximately 10^7 hybridoma cells in 0.5ml sterile PBS were injected IP into each mouse. Starting on day 25, ascites was collected, spun and frozen. A total of 72 ml was collected. This was re-spun, the aliquots from the different days mixed and frozen in 6 ml aliquots. The antibody was purified using saturated ammonium sulfate, PD-10 (Sephadex G-25m) and QAE. The final protein concentration was determined to be 9.1mg/ml.

Initial titering assays were performed to determine the optimal antibody and complement concentrations. The antibody is used undiluted : 100ul/10^6 cells at 4°C for 45 min. The cells are then spun, and resuspended in rabbit complement (Pel-Freez) diluted 1:3 in RPMI-1640 with 10% serum for 1 hr at 37°C.

Treated cells are then placed in growth medium (50% RPMI, 25% TCGF, 20% HL-1, 5% serum 0.125 mg/ml PHA and irradiated feeder cells at 50 x 10^6 cell/ml and dispensed at 200 μl/well (10^4 cells/well). Cloning plates are set up at 1, 2, 5 and 10 cells/well. Thioguanine plates are set up as for a usual *hprt* clonal assay (2 x 10^4 and 10^4 cells/well with 10^{-5} M 6-thioguanine). The plates are incubated at 37° C, 6% CO_2 for 10-18 days. Clones are expanded using growth medium as above. When clones are between 10-20 x 10^6 cells they are harvested:

1-3 x 10^6 cells are frozen in DMSO for future growth, 10^6 are treated for FACS analysis and 5-20 x 10^6 are frozen in $T_{10}E_1$ for DNA extraction and HLA Southern analysis. Cloning efficiencies and mutant frequencies are calculated as for the *hprt* clonal assay.

HLA and *hprt* mutant frequencies for 7 normal adults are shown in Table 12. The *hprt* and HLA assays were performed concurrently from the same blood sample and used the same cloning plates for Mf calculations. The frequencies for the *hprt* and HLA assays are generally fairly similar and are generally either both high or both low etc. in an individual. In two determinations, the antibody plus complement selection was incomplete and all HLA wells were positive for colony growth.

TABLE 12 - *hprt* and HLA mutant frequencies in 7 normal adults

Date	Individual	CE	hprt Mf	HLA Mf
11/29/89	KR	43.7	$9.5x10^{-6}$	$5.6x10^{-6}$
2/14/90	JD	39.6	$19.1x10^{-6}$	$5.3x10^{-6}$
3/14/90	DJ	23.8	$13.3x10^{-6}$	All pos
3/28/90	JS	30.1	$5.6x10^{-6}$	$1.0x10^{-6}$
4/25/90	ML	20.0	$8.3x10^{-6}$	All pos
5/2/90	KR	14.7	$20.1x10^{-6}$	$1.4x10^{-6}$
5/3/90	AB	33.7	$74.6x10^{-6}$	$23.8x10^{-6}$

The fluorescent activated cell sorter was used to assay the reaction of wild type and HLA mutant clones from individual AB using the anti-A2 monoclonal and FITC labeled anti-mouse second antibody. The results are shown in Table 13. (Background fluorescence for reactions without anti-A2 antibody ranged from 1-6% and have been subtracted out.) Since wild type clones ranged from 66-88% positive, those putative mutants with less than 60% positivity were assumed to be HLA A2 loss mutants (marked with *). These mutants are being analyzed by Southern blot analysis.

TABLE 13 - FACS results on putative HLA mutants

Wild type		HLA mutants	
clone	%positive	clone	%positive
4	66%	33	52%*
5	70%	35	50%*
6	84%	36	56%*
8	62%	37	33%*
9	88%	38	65%
12	76%	40	35%*
17	81%	42	17%*
		44	52%*
hprt mutant		46	54%*
82	84%	47	38%*
		48	62%
		49	26%*

Papers supported in whole or in part from DOE-sponsored Research (1990-1992):

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