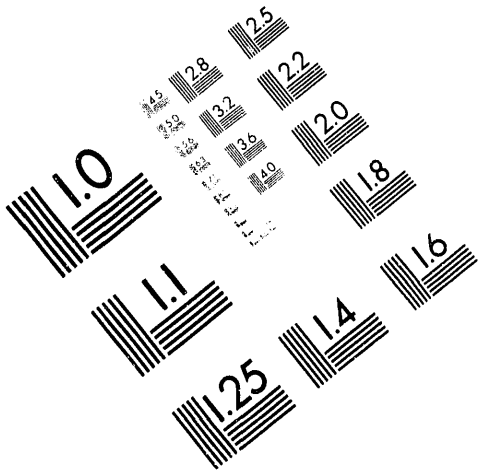

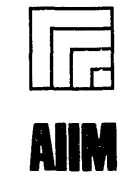

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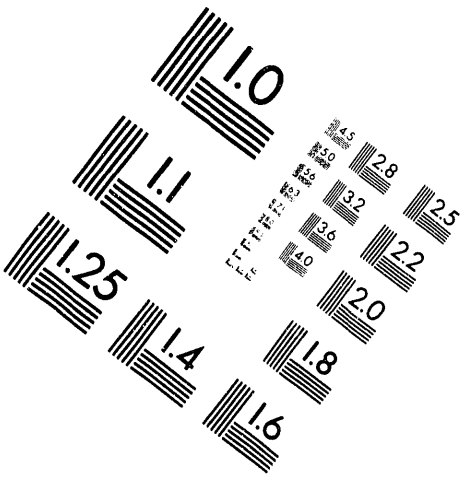

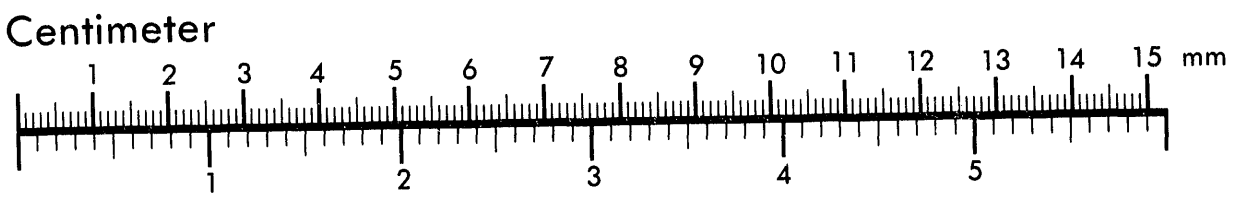

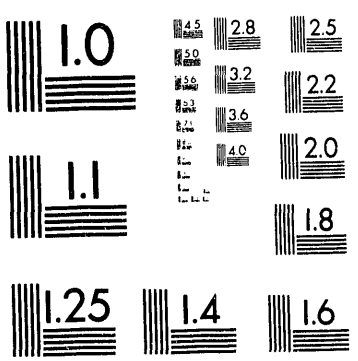

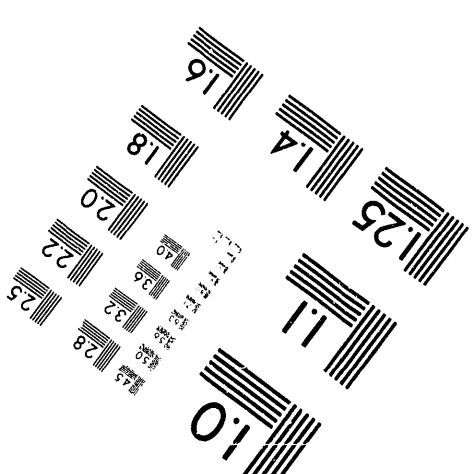

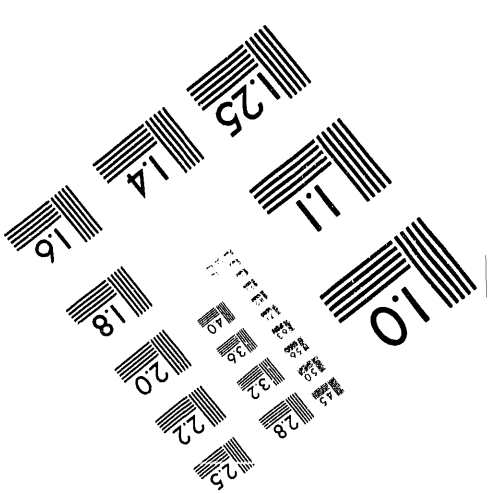

1 of 1

THE DEVELOPMENT OF IN VITRO MUTAGENICITY TESTING SYSTEMS USING T-LYMPHOCYTES

Research Progress Report: 11/01/92 to 10/31/93

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I. RESEARCH PROGRESS (11/1/92 to 4/30/93)

This research proposal included eleven specific aims. The research progress will be listed for each specific aim.

Work during the interval covered by this report has focused on Specific Aims #1, #2, #3, #8, #9 and #10 as presented in the grant application. Work on Aim #4 awaits receipt of mutant isolates from Dr. David Grdina, Argonne National Laboratory, who is doing the clonal assays and determining mutant frequencies for the clinical studies, as described in the application. The decision to reduce the effort on some specific aims was necessary because the grant award was less than requested to pursue work on all specific aims as described.

Specific Aim #1: Determine radiation induced mutant frequencies, clonality and molecular spectra for *hprt* mutations arising *in vitro* in human T-lymphocytes as a function of quality, dose and dose rate for the following ionizing radiations:

a. **γ radiation from a ^{137}Cesium source,**

b. **γ radiation from a ^{111}Indium source,**

c. **α particle and other high LET radiation from a radon source.**

During the past six months, we have focused on studies of the induction of *hprt* mutations after *in vitro* exposures to radon, in collaboration with Dr. Rick Jostes at Battelle Pacific Northwest Laboratories (PNL). Samples of human T-lymphocytes were exposed to radon at PNL and shipped back to the Genetics Laboratory for the mutation induction determinations. PNL also determined the precise dosimetry of the radon exposures (after performing radon/cell attachment assays with T-lymphocytes).

We have encountered variability in cell recovery after the radon exposures, primarily as a result of the time required for shipping, exposure and post exposure storing of the cells. The protocol entails drawing blood and isolating the T-lymphocytes on Monday and shipping to PNL, exposure to radon on Tuesday, storage of the cells for radiation decay until Wednesday and shipping back to the University of Vermont for receipt on Thursday. (We are developing alternative protocols for future use.) Using this procedure, we have initial results on the cytotoxicity (Figure 1) and mutagenicity (Figure 2) of radon exposure in T-lymphocytes in preliminary studies. These results demonstrate the feasibility of the study and we will now define the dosimetry of the mutation induction and isolate mutant clones for molecular analysis.

Specific Aim #2: Determine radiation induced mutant frequencies, clonality and molecular spectra for *hprt* mutations arising *in vivo* in human T-lymphocytes as a function of quality, dose and dose rate (thus constituting complete *in vivo/in vitro* studies) for the following ionizing radiations:

a. **γ radiation from a ^{111}Indium source,**

b. **α particle and other high LET radiation from a radon source.**

Specific Aim #8: Modify the currently used cloning assay for *in vivo* arising *hprt* mutations in human T-lymphocytes to allow an *in vitro* expression interval for recognition of *in vivo* DNA pre-mutagenic lesions requiring cell division for "fixation".

We have collected samples from cancer patients receiving ^{111}Indium conjugated antibody. We have measured the *hprt* mutant frequency arising *in vivo* and the mutant frequency after allowing for an *in vitro* expression of *in vivo* damage. The latter study employed the *in situ* method of phenotypic expression. The results are summarized in Table 1.

The first three samples assayed (RS, BD, and DU) showed no increase in the mutant frequency (Mf) as a result of the single treatment with ^{111}Indium (approx. 5.0 mCi). The absence of an increased Mf after the *in situ* expression suggests that there is no potentially mutagenic damage either. However, the more recent studies with two patients (AA and MG) shows increased Mf in samples collected post-treatment. We are not aware of differences among these five patients, which would explain these results. We will continue these studies as we obtain post-treatment samples. At present, we have pre-treatment samples from ten patients and are hoping to obtain two post-treatment samples from each.

Specific Aim #3: Completely characterize "large" *hprt* mutations in human T-lymphocytes (i.e. large deletions and other structural rearrangements which are the "signatures" of ionizing radiation induced *hprt* mutations), by:

a. **Characterizing the *hprt* gene region Xq26 using pulsed field gel electrophoresis and linked anonymous DNA sequences,**

b. **Defining deletion breakpoints,**

c. **Establishing maximum deletion sizes, and**

d. **Identifying *hprt* region Xq26 deletion size limiting factors (i.e. possible critical flanking genes).**

We wish to characterize the deletion breakpoint spectrum occurring in *hprt* flanking regions at Xq26 to uncover any deletion hotspots in this large chromosomal region. As a prelude to fine-mapping deletion breakpoints, we are physical mapping the *hprt* chromosomal region using pulsed field gel electrophoresis and a small number of *hprt* - linked probes. In the course of our physical mapping study, it became clear that further probe sequences near *hprt* were necessary to complete this work. We were successful this year in developing subcloning techniques from yeast artificial chromosomes (YAC's) with which to obtain greater probe coverage of the Xq26 region. The YAC's which contain large inserts containing human Xq26 sequence were a generous gift from Dr. David Schlessinger, Washington University, St. Louis. Briefly, the subcloning strategy is to isolate total YAC containing yeast genomic DNA (Figure 3). DNA is restricted with BamHI and ligated into plasmid pACYC184 (Figure 4). All recombinants are screened by Southern hybridization with a human repetitive sequence probe. Inserts from plasmid recombinants which are positive (i.e. contain human DNA) are isolated and restricted with Sau3AI to give multiple 0.5 - 2 Kb fragments. Some of these fragments contain the repetitive sequence and other fragments contain unique human sequence which was adjacent

to the repetitive sequence. We then screen Sau3AI fragments as to their utility as probes. Beginning with YAC yWXD837 which contains a 630 Kb insert containing *hprt* and extending telomeric, we have isolated four subclones: 837C-67I, 837C-19I, 837C-2I, and 837C-96I. At this point we have isolated two fragments from these subclones which hybridize to single copy human Xq26 sequence by Southern: 837C-96I-BA and 837C-67I-BC. We are currently utilizing these new probes in our physical mapping studies as well as applying the subcloning strategy to other YAC's mapped to different regions of Xq26.

We continue to fill in gaps of the Xq26 physical map shown below using the technique of pulsed field gel electrophoresis (Figure 5). Restriction fragment sizes (in Kb) are indicated. Dotted lines indicate regions where contiguous restriction fragments are missing. There may be small gaps in other regions of the map as well. Several *hprt* deletion mutants have lost both of the outermost markers indicating that they have survived deletion of greater than 2 Mb.

Specific Aim #4: Determine the induced mutant frequencies, clonality, and molecular mutational spectra of *in vivo* arising *hprt* T-cell mutants resulting from the treatment of cancer patients with the radiomimetic alkylating agent cyclophosphamide, with or without the radioprotector WR-2721 (S-2-[3-aminopropylamino] ethylphosphorothioic acid), and define differences in the molecular characteristics of mutants derived from the two treatment regimens that may be attributable to WR-2721.

Specific Aim #5: Determine the induced mutant frequencies, clonality and molecular spectra of *in vitro* arising *hprt* T-cell mutations resulting from the treatment with 4-hydroperoxycyclophosphamide, the self-activating metabolite of the radiomimetic alkylating agent, cyclophosphamide, with or without the radioprotector WR-2721 and with the radioprotector alone, and define the differences in the molecular characteristics of the mutants derived from the three treatment regimens that may be attributable to the WR-2721.

Specific Aim #6: Determine the induced mutant frequencies, clonality and molecular spectra of the *in vitro* arising *hprt* T-cell mutations resulting from treatment with γ irradiation with and without the radioprotector WR-2721, and define the difference in the molecular characteristics of the mutants derived from the two treatment regimens that may be attributable to the WR-272.

Specific Aim #7: Determine UV induced mutant frequencies, clonality and molecular spectra for *hprt* mutations induced *in vitro* in human T-lymphocytes as a function of dose for UV radiation.

We have not yet begun these studies. At present, we are analyzing *hprt* mutations induced by *in vitro* exposure to 300 cGy of gamma irradiation in order to define that mutation spectrum. Dr. David Grdina of Argonne National Laboratory has begun to do the Mf clonal assays on patients in this protocol. We anticipate receiving mutant clones for analysis during the coming year.

Specific Aim #9: Identify the cellular and genetic bases for inter-individual differences in susceptibility to radiation induced mutation/carcinogenesis in the general population by *in vivo/in vitro* T-cell *hprt* mutation studies of ataxia-telangiectasia (AT) and xeroderma pigmentosum (XP) homozygous and heterozygous individuals. These studies will include:

a. Defining *in vivo hprt* T-cell mutant frequencies, clonality, and molecular mutational spectra in constitutional homozygotes and heterozygotes for both disorders,

b. Identifying non-random distributions of *hprt* mutational events among *in vivo* T-cell clones in constitutional AT and XP heterozygotes as markers of intra-individual inter-clonal differences in susceptibility to radiation induced mutations in these individuals, and

c. Characterizing *hprt* mutant isolates representing the potentially hypersensitive *in vivo* clone(s) (derived from the constitutional AT and XP heterozygotes) for functional cellular homozygosity of the relevant gene (AT or XP), and determine if this functional cellular homozygosity arose *in vivo* in the heterozygote by somatic mutation of the single functional AT or XP allele.

We have focused on defining the *hprt* mutant frequency in AT heterozygotes and analyzing the mutant clones for mutation at the *hprt* locus, *in vivo* clonality and possible homozygosity for the AT locus by RFLP analysis. We have determined the mutant frequencies in samples from 8 AT heterozygotes (Table 2). Isolated mutant clones have been analyzed for the *hprt* mutations by Southern blot (Table 3) and cDNA sequencing (Table 4). The spectrum of *hprt* mutations is as yet too small for conclusions to be drawn. The analysis of the *hprt* mutant clones for TCR gene rearrangement pattern revealed no unusual *in vivo* clonality. However, we have observed two doublets in wild type clones, something not found previously in any study. We have no explanation at present for this observation. These clones will be analyzed further for TCR gene usage.

We have obtained six chromosome 11q22-23 probes for RFLP analysis of the region containing the AT gene(s). The hypothesis to be tested is that, in AT heterozygous individuals, mutation or recombination resulting in the loss of the single functional AT allele would yield a cell more susceptible to *in vivo* mutation. Such a cell population would significantly contribute to the *in vivo* mutant frequency. Our approach is to define the allelism for these chromosome 11 markers and then analyze mutant clones from individuals constitutionally heterozygous for the markers. A loss of heterozygosity would define a clone which has lost the linked AT allele through deletion or recombination. Such a clone would demonstrate the intra-individual interclonal differences and define a cell population more susceptible to *in vivo* mutation. The results to date are given in Table 5. We have begun to analyze clones with these probes to define those individuals who show RFLP differences consistent with chromosome 11 polymorphisms. At present, individuals heterozygous for one of these probes in wild type clones have also shown heterozygosity in all the mutant clones. These results may be due to the physical distance between the probe and the A.T. gene. Further studies with additional probes are ongoing.

Specific Aim #10: **Continue development of cloning assays for *in vitro* arising mutations in human T-lymphocytes occurring in autosomal genes, e.g. the HLA and adenine phosphoribosyltransferase (*aprt*) genes.**

We have focused on the development of an assay to measure mutations at the autosomal *aprt* locus in T-lymphocytes. We have obtained blood samples from individuals homozygous for *aprt* deficiency ($aprt^{-/-}$) and from their *aprt* heterozygous ($aprt^{+/-}$) parents. The conditions for cloning of the $aprt^{-/-}$ cells in the presence and absence of diaminopurine (DAP) have been defined. The conditions are essentially the same as used in the *hprt* cloning assay except for the use of an $aprt^{-/-}$ lymphoblastoid feeder cell strain (obtained from N. Drinkwater) and the use of 100 mM DAP to select for homozygous *aprt* deficiency. To test this selection system, cells from four *aprt* heterozygous ($aprt^{+/-}$) individuals and one normal ($aprt^{+/+}$) individuals were tested for cloning in different amounts of DAP. Concentrations of 50 μM or greater gave cloning efficiencies of less than 10-7 with cells from $aprt^{+/+}$ individuals and in the range of 10-150 x 10^{-6} with cells from $aprt^{+/-}$ individuals. These represent $aprt^{-/-}$ mutants and are presently being analyzed for the nature of the second *aprt* mutation. We have been unable to define an amount of DAP which allows cloning of $aprt^{+/-}$ cells but not $aprt^{+/+}$, i.e. we are as yet unable to select $aprt^{+/-}$ mutants arising in $aprt^{+/+}$ individuals.

Specific Aim #11: **Refine and automate the short-term assay for *in vivo* arising *hprt* mutations in human T-lymphocytes to allow more rapid population mutagenicity monitoring.**

We have not pursued this aim during this grant period.

PUBLICATIONS SUPPORTED BY THIS GRANT
DURING THE PERIOD 11/1/92 TO 4/31/93

Fuscoe JC, Zimmerman LJ, Harrington-Brock K, Burnette L, Moore MM, Nicklas JA, O'Neill JP, Albertini RJ (1992) V(D)J recombinase-mediated deletion of the *hprt* gene in T-lymphocytes from adult humans. Mutat Res 283:13-20

Albertini RJ, Nicklas JA, Fuscoe JC, Skopek TR, Branda RF, O'Neill JP (1993) *In vivo* mutations in human blood cells: Biomarkers for molecular epidemiology. Environ Health Perspect 99:135-141

Albertini, RJ (1993) Somatic Cell Gene Mutations in the Fetus: Measurement, Analysis and Potential Health Implications. In: Genetics and Development, Oxford University Press, New York, NY (in press)

Albertini, RJ, Nicklas, JA and O'Neill, JP (1993). Somatic Cell Gene Mutations in Humans: Biomarkers for Genotoxicity; Environmental Health Perspectives (in press)

O'Neill JP, Albertini RJ, Nicklas JA (1993) Molecular analysis of mutations induced *in vivo* in humans. In: Garte SJ (ed) Molecular Approaches to Environmental Science. Lewis Publishers, Inc., Chelsea, MI (in press) .

TABLE 1

IN VIVO HPRT MUTANT FREQUENCY IN PATIENTS RECEIVING ^{111}INDIUM CONJUGATED ANTIBODIES

Patient	Treatment (Time)	C.E.	MF x 10^{-6}	
			In Vivo	In Situ
RS	Pre	0.16	32.9	79.1
	Post (1 month)	0.11	28.5	19.0
BD	Pre	0.31	21.1	21.1
	Post (1 month)	0.80	11.8	13.1
DU	Pre	0.25	15.7	20.9
	Post (1 month)	0.29	19.8	16.2
AA	Pre	0.13	4.0	-
	Post (1 month)	0.08	15.7	-
	Post (2 months)	0.03	35.1	-
MG	Pre	0.09	5.8	-
	Post (3 months)	0.10	15.8	-
	Post (9 months)	0.08	19.8	-

TABLE 2

hprt MUTANT FREQUENCY IN A.T. HETEROZYGOTES

Individual	(Gender)*	Non-selected C.E.	Mf x 10^{-6}	Mutants for Analysis
LS-631A	(M)	0.43	25.6	4
LS-631C	(M)	0.58	6.6	4
LS-633A	(M)	0.55	10.8	24
LS-633B	(F)	0.72	12.9	37
LS-633C	(F)	0.51	12.6	1
LS-631IIB	(M)	0.75	10.8	8
LS-631IIC	(F)	0.52	2.5	8
LS-631IID	(M)	0.74	7.0	21
LS-637A	(M)	0.53	20.2	84
LS-637B	(F)	0.60	12.3	37
LS-637C	(M)	0.73	9.6	33

*M = Male, F = Female

TABLE 3

.D TYPE AND MUTANT CLONES FROM A.T. HETEROZYGOTES
I *HPRT* AND TCR GENE PROBES

RT MUTATIONS OBSERVED			TCR SETS
dIII	BamH1	Pst1	β & γ Probed Hind and Bam
change	No change		None
+3)	No change		None
+3),-4,-5-9	-6-9,-(2+3) -1,-(4+5)		
',+New	Not determined		Doublet WT4=WT6
change	No change		Doublet WT2=WT3
change	-(2+3)	No change	Doublet M6=M56
ew ~14kb	No change	No change	
ew ~14.5kb	No change	No change	
ew ~9.8kb	No change	No change	
ew ~13.5kb	No change	No change	
ew ~5.5kb	No change	No change	
ew ~9.8kb	No change	No change	
ew ~5.5kb	No change	No change	
ew ~9.8kb	No change	No change	
ew ~13kb	No change	No change	
ew ~5.5kb	No change	No change	
ew ~5.5kb	No change	No change	
ew ~9.8kb	No change	No change	
change		No change	Not determined
change		No change	Not determined

TABLE

SOUTHERN BLOT ANALYSIS OF WILD TYPE AND
WITH *HPRT* AND

SERIES	# WT CLONES	# MUTANT CLONES	HPRT MUTATIO	
			Mutant #	HindIII
LS-637A	2	63	M1	-5-9,-4,-(2+3)
			M35	-(2+3)+13kb
			M38	-5-9,-(2+3),-4
			M47	-5-9 +New ~17kb
			M54	(-5-9)
			M66	-(2+3)+New 7kb
			M71	-(2+3)
			M73	-5-9,-4,-(2+3)
			M74	-5-9,-4,-(2+3)
			M85	-(2+3)+New 5.4k

; (cont.)

MUTANT CLONES FROM A.T. HETEROZYGOTES
CR GENE PROBES

'S OBSERVED		TCR SETS
BamH1	Pst1	β & γ Probed Hind and Bam
	-7-9,-6,-4	
	-1,-2	Doublet M16=M45
	-7-9,-6,-4,-3,	Doublet M6=M41 - No Bam β+γ
	-2,-1	Doublet M10=M19 -No Bam β+γ
	-6+New Fragment	
	~6.5kb	
	-4	
	-3	
	-2+3+New ~2.4kb	
	-7-9,-4,-6,-2,-3	
	-7-9,-4,-6,-2,-3	
	-1+New ~2.1kb	
	-3	

TABLE 4: SEQUENCING RESULTS OF HPRT MUTANT T-LYMPHOCYTES FROM AT/

PROJECT	PERSON*	DOSE	EXPT	CLONE	MUTANT TYPE**	BASE PAIR	SEQ	BP CHANGE
AT HET	F		LS-633B	M11	F	207	AA(G) GGG GGG C	+G
AT HET	F		LS-633B	M4	D	-	-	-
AT HET	F		LS-633B	M16	F	342	AT()A AAG T	-A
AT HET	F		LS-633B	M22	D	-	-	-
AT HET	F		LS-633B	M26	D	-	-	-
AT HET	F		LS-633B	M28	B	419	ACT G(C)C AAA	G>C
AT HET	F		LS-633B	M38	B	3	AT(C) GCG ACC	G>C
AT HET	F		LS-633B	M46	D	-	-	-
AT HET	F		LS-633B	M50	D	-	-	-
AT HET	F		LS-633B	M51	B	617	GTT T(A)T GTC	G>A
AT HET	F		LS-633B	M63	B	542	GGA T(G)T GAA	T>G
AT HET	M		LS-633A	M2	B	331	TCA (G)CA GGG	A>G
AT HET	M		LS-633A	M3	F	27	GGC GTC GT()	-G
AT HET	M		LS-633A	M8	F	175	GGA (GG)CC AT	-G
AT HET	M		LS-633A	M12	B	611	AAT C(G)T GTT	A>G
AT HET	M		LS-633A	M13	D	-	-	-
AT HET	M		LS-633A	M21	?	-	W.T.	-
AT HET	M		LS-633A	M21	?	-	ACT (XXX X)AG	-GGA A
AT HET	M		LS-633A	M21	?	-	-	-
AT HET	M		LS-633A	M22	B	618	GTT TG(A) GTC	T>A
AT HET	M		LS-633A	M26	B	119	CAT G(A)A CTA	G>A
AT HET	M		LS-633A	M39	D	-	-	-
AT HET	M		LS-633A	M45	?	-	-	-
AT HET	F		LS-633B	M44	D	-	-	-
AT HET	F		LS-633B	M42	F	211	GGG (C)GC TAT	G>C

* F = Female, M = Male
** F = Frameshift, D = Deletion, B = Base Substitution, ? = Splice Mutation
*** NC = No change, N.D. = Not determined

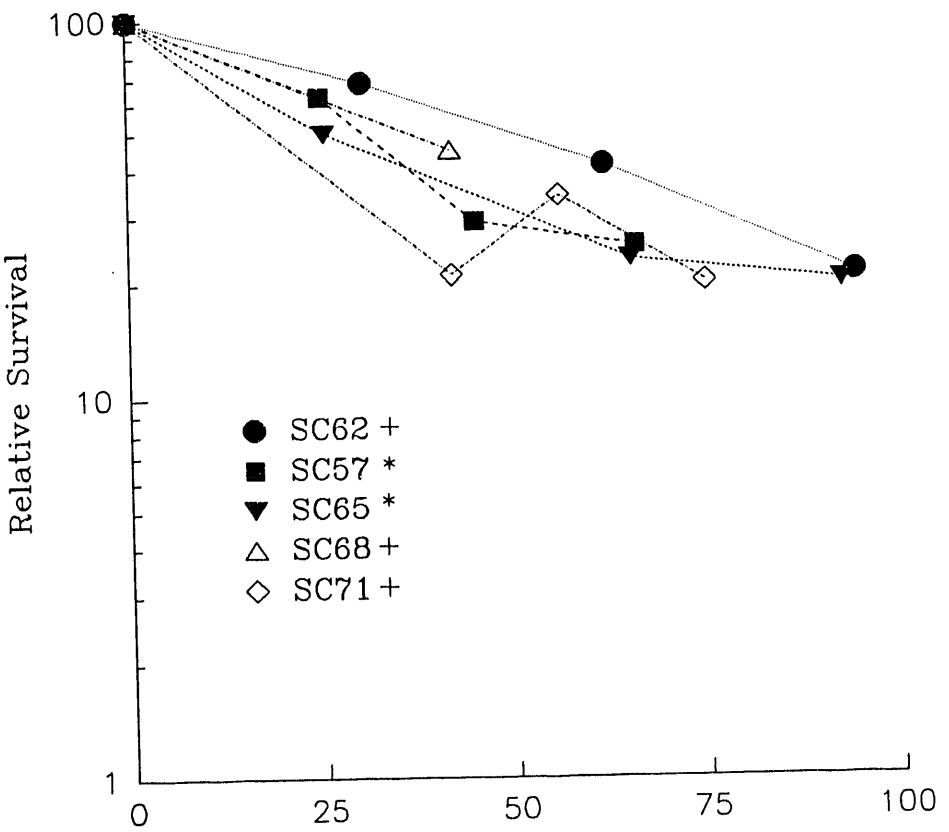
TABLE 5

USE OF CHROMOSOME 11q PROBES "LINKED" TO THE A.T. GENES FOR ANALYSIS OF A.T. ALLELISM IN T-LYMPHOCYTE CLONES

		11q Probes (Restriction Enzyme)		
Individual	Clones	STMY1 (TAQ 1)	D11S147 (Pst 1)	DRD2 (TAQ 1)
LS-631A	WT	Het*	Het	Het
LS-633B	WT	Het	Het	Hom**
	Mutant	Het (15)***	Het (19)	-
LS-633A	WT	Het	Het	Het
LS-631C	WT	Hom	Het	Hom
LS-631IIC	WT	Hom	-	Hom
LS-631IID	WT	Het	Hom	Hom
LS-637A	WT	Hom	Hom	Hom

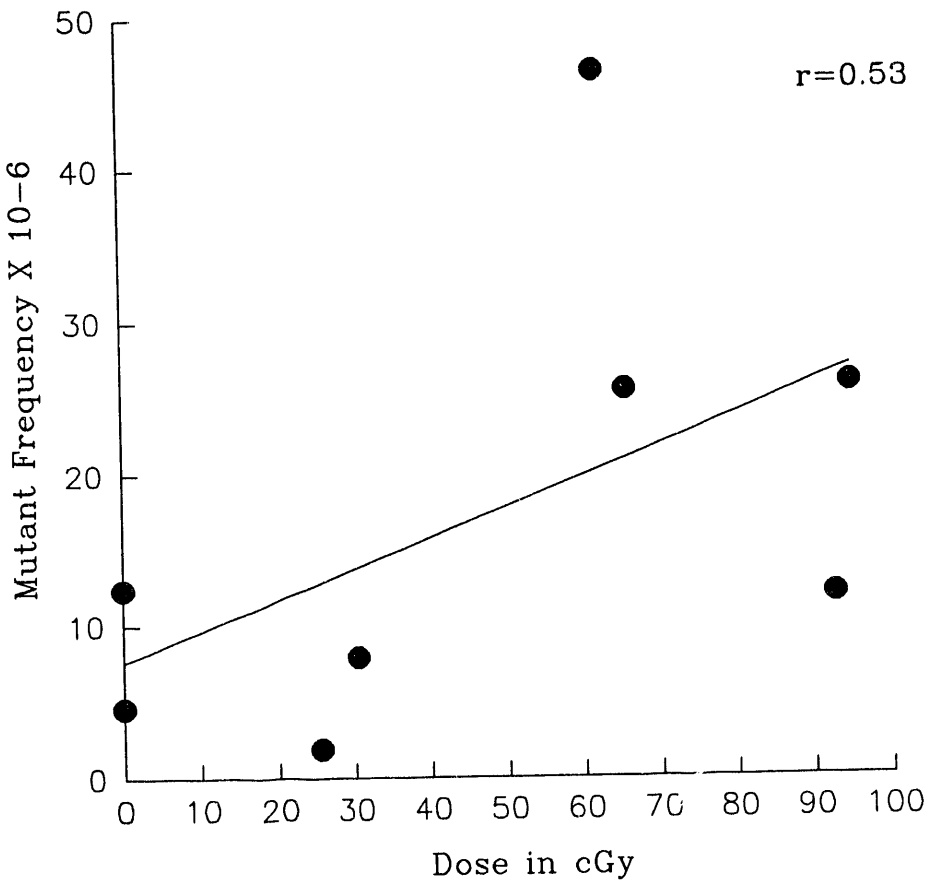
* Het = Heterozygous by RFLP analysis
** Hom = Homozygous by RFLP analysis
*** Number of clones analyzed

FIGURE 1



Survival curves for T-lymphocytes exposed to graded doses of radon and radon progeny
*=corrected doses
+=estimated doses

FIGURE 2



Relationship between mutant frequency and dose of T-lymphocytes exposed to radon and radon progeny.

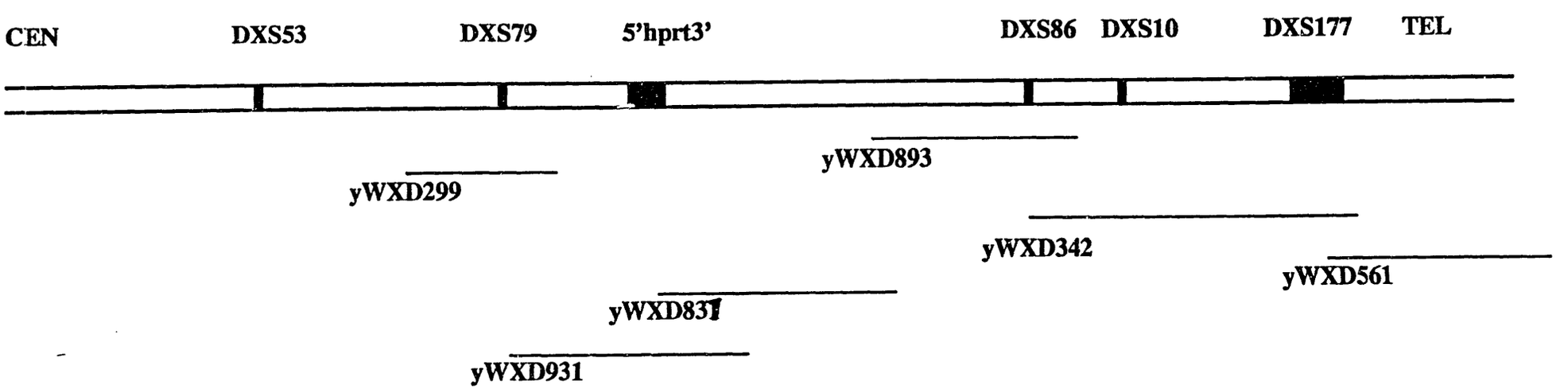
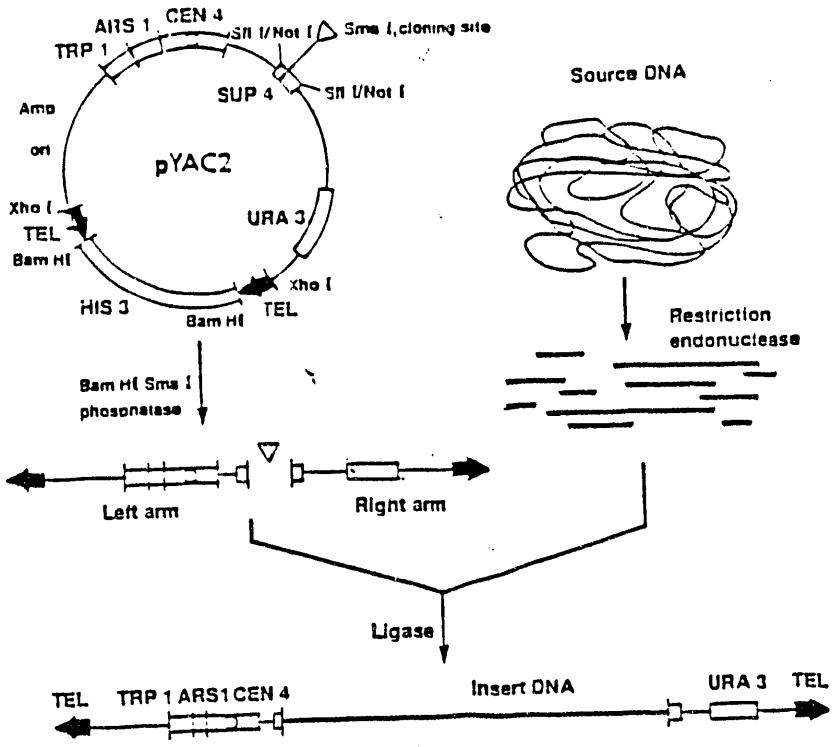


Figure 3 - YAC's with Xq26 Inserts

Figure 4 - Strategy to Obtain Xq26 Probes

The hprt chromosomal region that is tolerant of deletion is very large. To complete our studies, it is necessary to have greater probe coverage of this region. We received as a generous gift from Dr. David Schlessinger (Washington University) yeast artificial chromosomes (YAC'S) which contain human Xq26 insert DNA (see Figure 3).

Yeast Artificial Chromosomes



(Science 236:806-12)

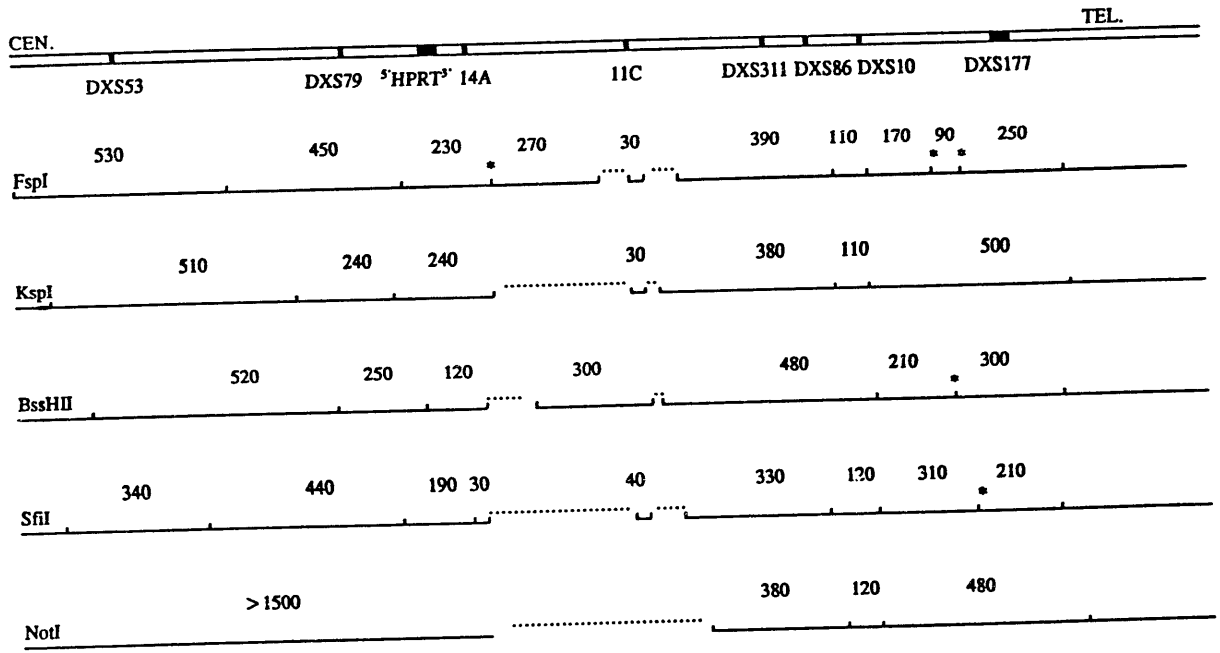
Strategy to Sub-clone Human Xq26 DNA from YAC'S into Plasmids

A) Isolate total YAC-containing yeast genomic DNA.

B) Ligate restriction fragments into plasmid vector and transform into E. coli.

C) Screen recombinants for those which contain human DNA by probing recombinants with human repetitive sequence containing probe.

D) Isolate Xq26 unique sequence DNA adjacent to the repetitive element in [illegible] sequences as probes.



* - partial digestion

Figure 5 - Xq26 Physical Mapping Studies

This schematic summarizes our physical mapping data. The Xq26 region is located at the top with a restriction map for each of the five enzymes used located below. Restriction fragment sizes (in Kb) are indicated. Dotted lines indicate regions where contiguous restriction fragments are missing. There may be small gaps in other regions of the map as well. Several hprt deletion mutants have lost both of the outermost markers indicating that they have survived deletion of greater than 2 Mb. We are currently engaged in obtaining further probes with which to complete the physical mapping studies. We will then search for deletion breakpoint "hotspots" within this region in our large collection of mutants.

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