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MOLECULAR AND CLONAL ANALYSIS OF *IN VIVO HPRT* MUTATIONS IN HUMAN CELLS

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INTRODUCTION

There is no longer doubt that gene mutations occur *in vivo* in human somatic cells, and that methods can be developed to detect, quantify and study them. Four assays are now available for such purposes; two detecting mutations that arise in bone marrow erythroid stem cells and two defining mutations that occur in T-lymphocytes (Albertini et al., 1982a; Albertini et al., 1988; Janatipour et al., 1988; Langlois et al., 1987; Morley et al., 1983; Stammatoyannopoulos et al., 1984; Turner et al., 1985, 1988; Strauss and Albertini, 1979). The red cell assays measure changes in mature red blood cells that involve either the blood group glycophorin-A locus (Langlois et al., 1987) or the hemoglobin loci (Stammayannopoulos et al., 1984); the lymphocyte assays score for genetic events at either the X-chromosomal hypoxanthine-guanine phosphoribosyl-transferase (*hprt*) locus (Albertini et al., 1982a, 1988; Morley et al., 1983; Strauss and Albertini, 1979; Turner et al., 1985) or at the autosomal HLA loci (Janatipour et al., 1988; Turner et al. 1988).

The concept of studying *in vivo* genetic changes in human somatic cells is not new. Attempts were made to develop quantitative assays for this purpose shortly after it was recognized that gene mutations could be detected in cultured mammalian cells (Atwood, 1958; Atwood and Scheinberg, 1958). A first assay was designed to detect loss of surface antigens of the ABO blood group from mature red blood cells. A second method attempted to recognize *in vivo* mutations of the X-chromosomal gene for glucose 6-phosphate dehydrogenase in polymorphonuclear white blood cells (Sutton, 1972, 1974). A third method was based on quantitation of fetal hemoglobin containing mature red blood cells (F-cells) in adults as indicators of hemoglobin beta gene inactivations *in vivo* in erythroid precursors, presumably as somatic events (Sutton, 1972; 1974; Stammatoyannapoulos et al., 1975; Wood et al., 1975).

Despite "reasonable" variant frequency values that sometimes even rose in an anticipated dose-dependent manner following exposures to environmental mutagens, all three of these assays had to be abandoned (Atwood and Petter, 1961; Papayannopoulos et al., 1977a, 1977b). The reason was the same for each, in that rare cells with the appropriate variant phenotype were found to arise from non-genetic causes (i.e. all of the systems were contaminated with phenocopies). Undoubtedly, some of the variants detected by these assays truly derived from *in vivo* somatic cell gene mutations; however, there were no means available to separate true mutants from phenocopies.

The failure of these three assays for human *in vivo* somatic cell gene mutation certainly points to one of the pitfalls that can be encountered when performing *in vivo* mutagenicity studies. Everyone engaged in such research now takes great pains to demonstrate the genetic bases of the target phenotypes. This has varying degrees of difficulty, depending on the system. However, a second problem, that of quantitating *in vivo* <u>mutation frequencies</u> from measured <u>mutant frequencies</u>, still receives little attention. Actually, the term "mutation frequency" continues to be used synonymously with the term "mutant frequency", although it is the latter that is usually determined, often without an obvious method for deriving the former.

We describe here our attempts to deal with both of these problems in a system when studying *in vivo* gene mutations in human T-lymphocytes.

The *hprt* T-cell Assays

hprt mutations arising *in vivo* in human T-lymphocytes are detected by two quite different methods. The first assay that was described uses an autoradiographic method to detect T-cells that can overcome 6-thioguanine (TG) inhibition of "first-round" lectin-stimulated DNA synthesis *in vitro* (Strauss and Albertini, 1979; Albertini et al., 1981, 1982b, 1988). The method allows enumeration of *in vivo* arising mutants and, as initially described (Strauss and Albertini, 1979), scored as "mutants" many cells that were not truly resistant to TG, i.e. scored for phenocopies. Thus, in its initial form, the autoradiographic assay had the same difficulties that forced abandonment of earlier assays. However, several methods became available to screen out the phenocopies; also the method of direct cloning (described below) allowed "verification" of the variant frequencies determined by the autoradiographic assay.

The short term autoradiographic assay remains useful because it is simple, relatively inexpensive, and has the potential for automation. All of these features are valuable for widespread human monitoring. Also, a modified method that uses BrdU incorporation followed by Hoechst staining (rather than autoradiography) to define TG resistant DNA synthesis has been described (Ostrosky-Wegman et al, 1987). The short term assay for TG resistant (TG^r) T-lymphocytes (i.e. *hprt* mutants) arising *in vivo* in humans is therefore available to any laboratory that is capable of performing SCE studies, allowing for its potential use in less developed areas of the world.

The fact remains however, that mutant cells cannot be isolated from these short-term mutagenicity assays for further analyses, thus negating some of the advantages of using nucleated blood cells for *in vivo* mutation studies. In order to remedy this, and to allow verification of the autoradiographic assay, a method employing direct cloning of *in vivo* arising T-lymphocytes was developed. This method permits isolation of the mutant cells, their *in vitro* clonal expansion, and characterizations at the biochemical and molecular genetic levels. Many laboratories have now used this clonal assay for quantitative and qualitative studies of *hprt* mutations that occur *in vivo* in human T-cells.

Heterogeneity of *in vivo* T-cell Populations

There is an enormous heterogeneity in the *in vivo* T-cell population that is manifest at many levels. For example, there are different functional classes of T-cells, e.g. "helper" or CD4+ lymphocytes, "cytotoxic/suppressor" or CD8+ lymphocytes, and subclasses within each. There is heterogeneity in "resting" versus proliferating T-lymphocytes, with the vast majority of cells in adults being in the resting, G_0 stage at any given time. In the extreme, there is the heterogeneity in the surface receptors present on mature T-cells. These T-cell receptors (TCR) confer the specificity of antigen reactivity.

When G_0 T-lymphocytes are activated *in vivo*, they undergo cell proliferation and clonal expansion. Activation occurs when a lymphocyte encounters antigen, (presented by antigen presenting cells) which is recognized by the TCR. These TCR's are dimeric proteins composed of α and β chains or, in some cells, γ and δ chains, and are associated with the CD3 antigen, creating a TCR-CD3 complex. The TCR genes encoding the TCR's, like those for immunoglobulins in B-lymphocytes, contain constant (C), variable (V), diversity (D) and joining (J) segments which undergo DNA rearrangement during T-cell differentiation to generate the diversity of the TCR's. It is

estimated that there are more than 10^7 different TCR gene rearrangement patterns possible. These different rearrangement patterns, as well as the unrearranged "germ-line" pattern, are readily recognized on Southern blots after appropriate DNA restriction analysis.

We exploit the TCR genes to characterize *in vivo hprt* T-cell mutations by correlating the specific TCR gene rearrangement pattern in a mutant isolate with its specific *hprt* alteration, and then comparing the combined TCR gene-*hprt* findings for a collection of mutant isolates from a given individual. This procedure is presented in detail elsewhere, and only the conclusions are given here.

When two or more mutant (or wild-type) T-cell clones from a given individual show the identical TCR gene rearrangement patterns following DNA digestion with two or more restriction enzymes and probing with two or more TCR gene probes (e.g. β and γ), we consider these clones to have originated from the same *in vivo* precursor mature T-cell. If the mutant isolates within such a group show, in addition, the same *hprt* change, we consider these to be replicate isolates of the same *in vivo hprt* mutational event (i.e. siblings). If the mutant isolates within a TCR defined group show different changes at *hprt*, these represent independent *hprt* mutations occurring in one *in vivo* expanding mature T-cell clone. On the other hand, when two or more mutant (or wild-type) isolates from a given individual show different TCR gene rearrangement patterns, the isolates originated from different *in vivo* precursors. If the *hprt* changes are also different, the various isolates represent independent *hprt* mutational events originating *in vivo* in different precursor mature T-cells.

Interpretation is ambiguous only in the case of two or more *hprt* mutant isolates from the same individual showing the identical change at *hprt* but different TCR gene rearrangement patterns. This is because TCR gene rearrangement itself occurs during the intra-thymic differentiation of T-cells, a process that occurs during fetal life and childhood, but which is largely completed during adolesence. Therefore, isolates with the last considered combined TCR gene-*hprt* pattern could represent a single pre-thymic *hprt* mutational event. Alternatively, the same combined pattern could represent repetitive, independent *hprt* mutations at a true mutational "hotspot" in different *in vivo* post-thymic mature T-cell precursors. One can resolve these alternatives however, if *hprt* mutational patterns are compared among individuals. True mutational hotspots should be seen in many individuals, whereas pre-thymic independent mutational events should differ among different individuals.

The analysis of *hprt* mutant T-cell isolates is aided greatly by the fact that there is apparent selection *in vivo* against *hprt* deficient T-cells. Thus, somatic mutational events usually occur in close temporal proximity to the recovery of the *hprt* mutant isolates. The results presented below have relied heavily on these types of analyses.

RESULTS

This section will summarize the evolution of T-lymphocyte assays from autoradiographic assay determinations of TG^r variant frequencies (V_f) through cloning assay determinations of TG^r mutant frequencies (M_f) to the final analysis of *hprt* mutation frequencies by use of TCR gene rearrangement patterns. In addition, the spectra of *hprt* mutations are being defined both for large deletions by Southern blot analyses as well as for small deletions/additions and base substitutions by DNA sequencing studies. Lastly, the combination of TCR gene rearrangement patterns with the *hprt* mutation spectra is allowing definition of the timing of mutation events at this locus in T-lymphocytes. The analysis can differentiate pre- or intra-thymic mutations from post-thymic mutations as well as those post-thymic mutations which occur in dividing versus non-dividing cells. This approach should elucidate the mechanisms of mutation induction in T-lymphocytes and the role of cell proliferation in that induction.

The autoradiographic assay (V_f)

This assay studies T-cells that are obtained from the mononuclear cell (MNC) fraction of heparinized whole blood by Ficoll-Hypaque density centrifugation (Boyum, 1968). For use in the autoradiographic assay, MNC's must be cryopreserved to eliminate the scoring of phenocopies, as discussed in detail elsewhere (Albertini et al, 1981). MNC's are therefore suspended in 7.5% dimethylsulfoxide (DMSO) and cryopreserved in the vapor phase of liquid nitrogen prior to test. For test, MNC's are thawed, stimulated with phytohemagglutinin (PHA), cultured in multiple flasks with or without $2x10^{-4}$ M TG for 24 hours, labeled with tritiated thymidine and cultured for an additional 16 hours. Measurements can be made by two methods, a labeling index method or a dilution method. These involve either differential counts of labeled and unlabeled nuclei (labeling index method; usually a total of 5000 nuclei) or the enumeration of total labeled nuclei in diluted cell samples to yield a measure of lymphocyte labeling in the absence of TG. Either the frequency or the number of nuclei labeled in the presence of TG divided by the labeling index or the number of labeled nuclei corrected for dilution in non-TG cultures, respectively, yields the V_f. Both methods yield similar V_f values (Albertini et al, 1988); however the dilution method is more amenable to automation.

Determinations of V_fs in normal adults (age 30-62; 82 assays on 26 individuals) yielded a mean (± SD) value of 8.7 (± 6.1) x 10^{-6} (Albertini, 1985; Albertini et al, 1988). Increases in V_f were found in patients exposed to chemotherapeutic agents or ionizing irradiation (Albertini, 1985). This assay clearly allows measurement of the frequency of TG^r variant cells and is useful for monitoring studies. As noted, the recent development of a BrdU labeling procedure should expand the utility of this short term assay (Ostrosky-Wegman et al, 1987). However, interpretation of the V_f values is hindered by the inability to confirm the mutant nature of the TG^r labeled cells. This deficiency was removed by the development of a T-lymphocyte cloning assay.

The cloning assay (M_f)

The cloning assay can be performed on either fresh or cryopreserved MNC's. It too has been described in detail (O'Neill et al, 1987, 1989) and involves the direct cloning of peripheral blood MNC's (either fresh or cryo-

preserved) *in vitro* in limiting dilutions (1-2 cells/well, non-selective conditions; and $5x10^3$, $1x10^4$, or $2x10^4$ cells/well in 10^{-5} M TG) to define cloning efficiencies in the presence and absence of selection. Interleukin-2 (IL-2) and irradiated human lymphoblastoid cells are required for cloning and cell growth. Cloning efficiencies are calculated from the Poisson relationship $P_o = e^{-x}$, where x is the average number of clonable cells/well. A mutant frequency for an individual sample is the ratio of the cloning efficiency with TG to cloning efficiency without TG. Both wild type and mutant colonies are isolated from the wells of microtiter plates, and expanded in medium containing IL-2 and lymphoblastoid feeder cells to populations of sufficient size for phenotypic and genotypic characterizations.

Cumulative M_f values (mean ± SD) for 115 assays of 27 individuals (age ~ 20-35 years) were 6.5 (± 4.8) $x10^{-6}$ (Albertini et al, 1988). Thirty-three parallel determinations by both the autoradiographic and cloning assays (including some other than those included in the above averages) yielded V_f and M_f values of 8.4 (± 8.5) $x10^{-6}$ and 10.5 (± 6.3) $x10^{-6}$ respectively (Albertini et al, 1988). The similarity of these values suggests that the V_f measurement does reflect the actual M_f value, and validates the V_f measurement for population studies.

The reproducibility of the cloning assay performed as described in O'Neill et al, (1987) was assessed in a longitudinal study of 6 normal individuals (3 male, 3 female, age 22-33 years) employing 4-5 samples over a 26-37 week time period. No significant differences were found among the M_f values and the mean (± SD) values for 46 assays was 5.4 (± 2.4) $x10^{-6}$ (O'Neill et al, 1989).

In addition to these studies with young adults, we have investigated the M_f in two other age groups. To assess the *hprt* mutations during fetal development, placental cord blood samples have been utilized. The mean (± SD) M_f for 45 samples (38 newborn male, 7 newborn female) was 0.64 (± 0.41) $x10^{-6}$ (McGinniss et al, 1989a). This result demonstrates a quantitative difference between fetus and adult. Further evidence for an age related increase in M_f was found in studies of older individuals (55 years and older). In this group the mean (± SD) M_f was 16.5 (± 6.4) $x10^{-6}$ for samples from 28 individuals.

Determination of the mutational basis of TG^r T-cells

To fully validate the cloning assay as a measure of mutation at the *hprt* gene, and not as a detector of phenocopies, it is necessary to demonstrate loss of HPRT enzyme activity and/or mutational changes in the *hprt* gene. To date, all tested TG^r colonies have maintained TG resistance after growth in the absence of TG and have shown loss or reduction in HPRT enzyme activity (Albertini et al, 1982; Albertini, 1985). Additional evidence for the mutational nature of these TG^r colonies lies in the molecular analysis of the *hprt* gene alterations observed in Southern blot and sequencing studies. All TG^r mutants thus far analyzed show specific *hprt* gene mutations (see below).

TCR gene rearrangement patterns to define mutation frequencies

The cloning assay provides a quantitative measure of TG^r mutant frequencies in human T-lymphocytes. However, the goal of this research is to determine *hprt* mutation frequencies. This can be accomplished by taking advantage of the rearrangements of TCR genes, as outlined above. These TCR gene rearrangements can be uniquely employed as independent measures of

clonality of isolated T-cell colonies, and used to determine mutation from mutant frequencies, as described in detail in Nicklas et al, (1986).

The TCR gene probes have been used to analyze 94 wild type (unselected) and 326 *hprt* mutants (TG selected) from three individuals (Nicklas et al, 1989) whose mutant frequencies were measured in the longitudinal study described above (O'Neill et al, 1989). All the wild type colonies showed different TCR gene rearrangement patterns, indicating that they derived from independent TCR defined *in vivo* precursors. Approximately 90% of the mutant colonies showed unique TCR gene patterns, demonstrating that the measured mutant frequencies often closely approximate actual mutation frequencies (Nicklas et al, 1989), at least in normal young men with "normal" M_f values. These results are summarized in Table 1 where the distribution of TCR gene defined "sets" from given individuals is also shown. The significance of these TCR sets will be discussed below. The initial analysis was performed primarily to more closely approximate the mutation frequencies from the measured mutant frequencies.

These detailed studies with 3 normal individuals suggesting that *in vivo* mutant frequency values are close approximations of *in vivo* mutation frequencies tend to confirm our earlier results obtained in more limited studies with 8 other normal individuals where 137/154 (89%) TG^r mutants showed different TCR gene patterns (Nicklas et al, 1987). However, there are exceptions, primarily in individuals who show elevated *in vivo* mutant frequencies. Table 2 shows results from 3 individuals who are considered "outliers" in that the measured mutant frequencies exceed 50×10^{-6}. Individual G is an apparently normal woman whose mutant frequency has shown a progressive rise over a 4 year period. Repeated analyses by Southern blots of the TCR gene patterns in these mutants isolated from several different blood samples have revealed that approximately 98% of them are identical (Nicklas et al, 1988). In the results shown in Table 2, 94% of the mutants from one blood sample were in the single TCR gene defined set. Thus, this individual has a greatly elevated *in vivo* mutant frequency, but this is not due only to an increased mutation frequency but mostly to *in vivo* clonal expansion of one TCR gene defined progenitor. Any *hprt* gene mutation (or mutations) that occurs in this expanding cell will be greatly amplified, thus distorting the relationship between mutants and mutations. This shows the importance of defining mutation frequencies directly, and not assuming them to be synonymous with mutant frequencies. Table 2 lists two other examples of *in vivo* clonal expansion of TG^r mutants. Individual H is a patient with chronic active hepatitis who was treated with the immunosuppressive agent azathioprine. The increased mutant frequency is not surprising since this agent is metabolized to 6-mercaptopurine *in vivo*, which might result in *in vivo* selection for TG^r resistance. Limited studies of 32 mutants with 1 restriction enzyme and 2 TCR probes reveal that 13 of 32 mutants have the same TCR gene pattern. This substantial fraction of mutants that show the same TCR gene patterns means that the mutation frequency is most likely much lower than the measured M_f. Individual I is a patient with ovarian cancer, whose *hprt* mutant population shows that a large number share the same TCR gene rearrangement pattern after study with 2 enzymes and 2 TCR gene probes. Again, the actual mutation frequency may be much lower than the measured mutant frequency. The conservative estimate of mutation frequency, in the absence of information concerning the actual molecular changes in the *hprt* gene, is to consider all

mutants that share the same TCR gene pattern to have derived from a single *in vivo hprt* mutational event.

These three examples demonstrate the variable relationship between measured mutant frequencies and their underlying mutation frequencies. Approaches must be developed to assess the contribution of *in vivo* clonal expansion of TG^r mutants to an elevated mutant frequency value. TCR gene rearrangement pattern analysis is able to supply this information.

Spectrum of *hprt* gene mutations

The ability to propagate T-cell mutant colonies *in vitro* allows analysis of the mutations in the *hprt* gene. The initial studies employed Southern blot procedures to define the large structural alterations such as gross gene deletions and rearrangements. Early studies of 164 mutations from 8 normal males showed alterations detectable by Southern blots in 16 colonies (9.8%) (Nicklas et al, 1987). A larger study has now been completed with 326 mutant colonies from 3 individuals (Nicklas et al, 1989). The mutant frequencies for these individuals are listed in Table 1 with the fraction of mutants and mutations containing structural alterations by Southern blot analyses. The profiles of these alterations are shown in fig. 1. Of the 38 mutations (45 mutants) with detectable *hprt* gene alterations, 28 are simple deletions (12, 9 and 7 from D, E, and F respectively: the open boxed area mutants in Fig. 1). Individuals E and F show replicate isolates of the same mutational events in two or more mutant colonies, shown by superscripts 1, 2, 3 and 4.

This subgroup of mutations allows analysis of the breakpoint distribution patterns for *hprt* gene deletions in these 3 adults. The breakpoints are designated as occurring in introns or the 5′ or 3′ flanking regions for deletions extending beyond the gene (arrows in fig. 1). The breakpoint distributions are shown in Table 3 with the *hprt* gene divided into 3 approximately equal size pieces. Several conclusions can be drawn from these data. Breakpoints are distributed evenly across the gene in terms of length suggesting no "hotspot" for this type of mutation. Based on this even distribution and the total number of breaks determined to lie within the gene (25/55), the approximate size of deletions extending into flanking regions can be calculated. This does assume a probability of extragenic events equal to the frequency of intragenic events (25 breakpoints per 43 kb of DNA). The 13 breakpoints 5′ to the gene and the 17 breakpoints 3′ to the gene predict an average size deletion of 22 kb 5′ and 29 kb 3′ to the *hprt* gene by this analysis. This suggests that X-chromosomal deletions involving *hprt* of at least 94 kb (43 + 22 + 29) can be recovered in human T-lymphocytes.

One interpretation of the fact that this calculation can even be made (that is, only 9 of 38 mutations resulted in total *hprt* gene deletion) is that the size of deletions involving this X-chromosome gene is limited because of some vital function gene which is closely linked. Deletion size may be restricted due to the hemizygous nature of the X-chromosome (structural in males, functional in females). In order to investigate this aspect of *hprt* mutation events, we are employing X-linked DNA probes for deletion mapping studies. Our initial studies used two anonymous X-chromosome probes, DXS10 and DXS144, in Southern blot analyses to determine co-deletion in *hprt* deletion mutants. The PstI digests from 4 wild type, 5 total *hprt* deletions mutants and 3 partial *hprt* deletion mutants were probed and the result is shown in fig. 2. Two of the 5 total *hprt* deletion mutations also showed loss of the DXS10 locus fragment. Estimates of the genetic distance between *hprt*

and DXS10 have ranged up to 14 cM, indicating that rather large deletions of the human X-chromosome can be recovered as viable T-cell *hprt* mutants which arose *in vivo*. Clearly these results indicate that recoverable deletion size per se is not a limitation in the analyses of *hprt* mutation in human nor to the use of such an assay for mutagenicity monitoring in adults.

We hope to develop a map of the *hprt* region using X-linked probes and our collection of *hprt* deletion mutants. We will then use pulsed field gel electrophoresis to define the size of deletions through shifts in fragment size of linked but undeleted markers.

The above approach has allowed analyses of *hprt* deletion and rearrangement mutations in normal adults. However, Southern blots have been too insensitive to detect *hprt* alterations in 86% of the mutants. Thus we have now begun sequencing studies employing reverse transcriptase to synthesize a cDNA to the *hprt* mRNA, and the polymerase chain reaction, for *hprt* cDNA amplification (Simpson et al, 1988). Thirty mutants from individual E have been sequenced to date. Twelve different single base substitutions (6 transition and 6 transversion mutations) have been found, including an identical base substitution in the five *hprt* mutant clones of the nonamer TCR gene set which have been sequenced. These are clearly *hprt* sibling mutants that derived from a single *in vivo* mutation. Other *hprt* mutations involve additions or deletions. The deletions include a variety of single exon deletions, probably due to splice site mutations. As found with the larger alterations, no site or type of *hprt* mutation predominates. These ongoing studies will define the total spectrum of "background" *hprt* mutations occurring in adults.

In an attempt to place the above spectrum of background *hprt* mutations observed in adults in perspective, we have also begun to characterize the *hprt* mutations that occur during human intra-uterine development as reflected in placental cord blood samples from newborns (McGinniss et al 1989a; 1989b). Thus far we have analyzed 41 mutant colonies from 13 normal male newborns. In striking contrast to the pattern found in young adults (in addition to the 10 fold lower mutant frequency) 35 of the 41 mutants (85%) showed *hprt* alterations on Southern blot studies. Twenty of the 35 mutants showed deletion of only exon 2 and 3, with some also showing a decrease in the size of the exon 1 fragment. When the TCR gene rearrangement patterns were analyzed as described below to define independent mutations (in this case pre- or intra-thymic mutations appear to predominate), these 41 mutants appeared to result from 31 independent mutations with 26 (85%) showing structural alterations. Thirteen of these 26 (50%) contained deletions of exon 2 and 3. The distribution of breakpoints in the 22 of 26 mutations which were simple deletions is listed in Table 3. (It must be emphasized that 8 of the 13 exon 2 and 3 deletion mutants contained a normal molecular weight (MW) exon 1 band while the other 5 contained a smaller MW exon 1 band. The breakpoints in intron 1 are clearly different in 4 of these latter 5 mutants.) Other mutants also showed breakpoints in intron 1 and intron 3 resulting in 31 of 44 breakpoints in intron 1 or intron 3 (one exon 2 deletion breakpoint was in intron 2). In fact, of the 33 intragenic breakpoints, 31 (94%) occurred in these two introns in the newborn, in sharp contrast to the more linear intron distribution observed in the adult (19 of 29 (65%) reside in intron 2 and 3). Clearly, specific deletion mutations of exon 2 and 3 predominate in the newborn. Only 25% of the breakpoints were extragenic (compared to 52% in the

adults) and most were mutations with 1 intra- and 1 extra-genic breakpoint (only 1 total exon 1-9 deletion was found).

There are at least two interpretations for these observations. One is that large deletions are not recovered from newborn samples because of closely linked vital genes important for T-cell survival during fetal life. These vital genes are less essential during the post fetal period perhaps because of the decline in cell proliferation; therefore, more large deletion mutations can be recovered in adults. (This would be a vital gene only *in vivo* since large deletion mutants clearly proliferate *in vitro*.) Alternatively, one might propose that the intragenic breakpoints in intron 1 or 3 are the result of a unique mechanism operating only in fetal lymphocytes either due to active proliferation or differentiation and that these events mask the other mutation mechanisms which also occur in newborns in common with adults. This would imply that the frequency of extragenic breakpoints, i.e. a total of 11, is much higher than the frequency of intragenic breakpoints not in intron 1 and 3 i.e. 2 (1 in intron 2 and 1 in intron 4 or 6) in the newborn. This latter interpretation suggests that deletions with extragenic breakpoints can occur at higher frequencies in newborns than in adults, and predicts a larger average size deletion involving the *hprt* locus in the newborns. From the viewpoint of deletion analyses, the study of cord blood samples is clearly valuable and may be a unique tool for X-chromosome deletion mapping in the human.

The use of combined TCR gene rearrangement patterns and specific *hprt* mutation analyses to define the timing of mutation events

We can employ analyses of the TCR gene rearrangement patterns to characterize the timing of *in vivo* T-cell mutations by correlating the TCR gene rearrangement patterns with the specific alteration responsible for the mutation in any mutant isolate i.e. the Southern blot or sequence change at the *hprt* locus. This approach will uniquely allow definition of mutational "hotspots" because it can differentiate between repetitive identical mutation and the clonal expansion of a particular mutation. Both will yield an increased frequency of mutants with the same mutation i.e. an identical effect is observed from either cause. Any study which purports to define the *in vivo* mutational "hotspots", mutagen specificity or any other aspect of a mutation spectrum must be capable of such differentiation. The approach with T-cells is based on the TCR genes as independent, unselected markers of clonality. At present, in addition to the definition of independent mutations, we are beginning to develop insights into the timing of mutation *in vivo* in humans.

The TCR gene analysis with mutants from newborns has allowed some insight into the timing of mutation during fetal development. Our initial molecular studies with newborns were limited to a small number of mutants from each of 13 cord bloods (total of 41 mutants) analyzed: 3 samples with 1 mutant, 3 with 2, 3 with 3, 1 with 4, 1 with 5, and 2 with 7 because of the low mutant frequencies seen in newborns. The results with 13 samples allowed us to propose exon 2+3 deletion as a "hotspot" in fetal development because 12 of 13 individuals show this mutation in 1 or more samples (clones from 6 individuals showed only the deletion, 4 showed only the deletion plus the exon 1 MW change and 2 showed both types of alterations).

In 5 individuals, the same *hprt* alteration was seen in 2 or 3 mutants with different TCR gene rearrangement patterns. This is the expected result for a pre- or intra-thymic mutation, i.e. a mutation which occurs in an

unrearranged stem cell prior to TCR gene rearrangement in the thymus. This finding leads us to suspect that TCR gene rearrangement mechanisms may result in low frequency, sequence specific rearrangements of other genes during the differentiation process. (The observation that these 5 samples display the putative exon 2+3 deletion "hotspot" mutation might dampen enthusiasm for this proposal on the grounds that "hotspot" mutations might equally occur repetitively in post-thymic cells to yield the same effect, i.e. same *hprt* mutation and different TCR gene patterns means post-thymic, independent events). However, two other samples also showed 2 mutants with the same *hprt* alteration and different TCR gene patterns, but this time with unique *hprt* changes (that is, 2 with exons 4-9 deletions and 2 with exon 2 deletions) which were not seen in other newborns. This is certainly consistent with pre- or intra-thymic mutation. Because of these observations, these 7 samples were considered to each represent a single *hprt* mutation for the breakpoint analysis discussed earlier. If these are not pre- or intra-thymic mutations, the magnitude of the intron 1 and 3 "hotspots" increases because those 11 mutants in the 5 samples were said to represent only 5 mutations, and the other 2 samples to represent only 2 and not 4 mutations.

A pre- or intra-thymic mutational "hotspot" in the fetus should be represented in the adult spectrum if the selection *in vivo* against *hprt* mutants is not absolute. There is, of course, some selection, because Lesch-Nyhan heterozygotes show mutant frequencies of 1-5 $x10^{-2}$ instead of the expected 0.5, a reduction of 10-50-fold over expectation (Albertini and DeMars, 1974; Strauss et al., 1980). We have observed exons 2+3 deletion mutations in adults in both individual D (mutant 9) and E (mutants 6-9, 11, and 12, fig. 1). Interpretation of this finding awaits more complete development of the spectrum of deletion mutations in adults. However, it is intriguing that of the six exons 2-3 deletion mutants observed in individual F, four of these (mutants 6-9) share both TCR β and γ patterns, and are *hprt* mutant siblings. The other two share a different TCR β pattern but each has a unique TCR γ pattern. This could be explained as the result of 1 pre-thymic mutation followed by proliferation and differentiation to yield three mutants and the subsequent TCR gene rearrangement. The four TCR siblings would be the result of the clonal expansion of one of these *hprt* mutant progenitors after TCR gene rearrangement.

Regardless of the time of mutation occurrence, we interpret the above cohort of four *hprt* mutants sharing a TCR gene pattern as an example of *in vivo* clonal expansion of a pre-existing mutant cell. Another example might be the nonamer seen in individual E where all five *hprt* mutant isolates thus far sequenced have the same base substitution mutation. Two sets of mutant isolates with the same TCR gene pattern and identical *hprt* alterations were seen in individual E: three exons 5/6-9 deletion mutants (mutants 5, 6 and 7, Fig. 1) and two exons 4-6 deletions (mutants 12 and 13, Fig. 1). (The other groups of mutant isolates that share TCR gene rearrangement patterns listed in Table 1 have not yet been analyzed for the *hprt* changes by DNA sequencing.) While the 5 mutants of the nonamer can be simply interpreted as a clonal expansion of a pre-existing mutant, interpretations of the other two TCR gene identical mutant sets are not so clear. A fourth mutant from individual E (mutant 10) shares both TCR β and γ patterns with mutants 5-7 but has a different *hprt* mutation (rearrangement, one breakpoint in exons 7-9). In addition, the two exon 4-6 deletion mutants share TCR gene rearrangement patterns with another mutant with a different *hprt* alteration (mutant 11, fig.

1). These findings suggest that mutations might be occurring in proliferating cells because of the existence of different *hprt* mutations in the same TCR gene defined T-cell clone.

Clones with a high rate of cell proliferation might be a more sensitive target for mutation per se, with repeated mutation events possible as proliferation continues. If proliferating T-cells are more "mutable" (because of DNA replication errors or decreased time for DNA repair prior to DNA replication) than are non-proliferating T-cells, isolates with the identical TCR gene rearrangement pattern may represent clones which have undergone recent *in vivo* cell division. It may not be that pre-existing *hprt* mutant clones proliferate to yield TCR defined sets of isolates, but that a specific TCR gene defined cell proliferates *in vivo* and, as a result of proliferation, *hprt* mutations occur. The *hprt* mutant might then continue to divide as part of the TCR defined proliferative response. Extensive proliferation might result in repetitive mutation in the TCR gene defined clone yielding the above observation of TCR gene defined sets of mutants with different *hprt* mutations. TCR gene defined sets of mutants might then reflect extensive cell proliferation and the spectrum of mutations in these cells reflect DNA replication related events. These replication related mutations might form a spectrum distinct from that found in non proliferating T-cells.

The extreme case of a proliferating T-cell population appears to have occurred in individual G (Table 2). Greater than 98% of the mutants show identical TCR gene rearrangement patterns. These TCR gene identical isolates may reflect a unique subset of mutations in human T-cells. Results of DNA sequence studies with these mutants are incomplete, but do show changes consistent with repetitive *hprt* mutations in this *in vivo* proliferating clone.

Analyses of mutants from newborns and adults may allow development of spectra of *hprt* mutations induced through 3 distinct mechanisms: (1) T-cell differentiation through TCR gene rearrangement and the persistence of these mutants in the adult (2) mutations occurring in primarily non-dividing cells in the adult and (3) mutations arising in cells that are actively proliferating *in vivo*.

SUMMARY AND FUTURE STUDIES

The methodologies described here clearly allow measurements of *in vivo* mutant frequencies in human T-lymphocytes and are now available for human monitoring. One approach to their use for large scale population studies may be to use the autoradiography or differential staining short term V_f assay as an initial screening assay because of its relative simplicity and potential for automation. Individuals with increased V_f values may then be confirmed with the cloning assay, employing cryopreserved aliquots of the original blood sample. This more demanding method would then be employed only where it makes its unique contribution, i.e. in verifying the mutational basis of the TG^r cells, for characterizing mutational spectra and, of equal importance for determining clonality. The cloning assay permits the specificity and mechanisms of mutation induction to be studied in humans.

Quantification of mutation (as distinct from mutant) frequencies can be accomplished by use of the TCR gene probes. This is the unique advantage of assays which employ T-lymphocytes. As outlined above, TCR gene rearrangement pattern analyses have revealed the existence of replicate copies of a single *hprt* mutation in TCR gene defined mutant sets. This observation has exciting implications for the understanding of mutation mechanisms in humans. Our current hypothesis is that the existence of TCR gene mutant sets in an individual is the result of mutation occurring in a proliferating T-cell population. We propose that, in response to an antigen (or other unknown stimuli), a limited cohort of TCR specific T-cells is stimulated and cell proliferation occurs. The probability of mutation is greater in this minority population of dividing cells than in the majority population of non-dividing G_0 phase cells and is proportional to the number of cell divisions. Therefore, the probability of a mutation occurring increases with the magnitude of the clonal expansion. A mutant cell, once induced, will continue to proliferate until the antigen stimulation ends. The existence of TCR gene defined mutant sets may then reflect expansion of antigen specific T-cells. Analysis of these mutants will provide information on mutation mechanism as well as T-cell antigen specificity. It might be possible to analyze antigen specificity and the immune response through analysis of TCR gene defined mutant sets. Elevated mutant frequencies due to an increase in TCR gene defined mutant sets may become an important measure of the *in vivo* function or dysfunction of T-lymphocytes.

Although TCR gene defined mutant set analysis offers a unique approach to understanding the mutation processes in humans, the very phenomenon being investigated poses a potential problem in interpreting mutant frequency values in studies designed for genetic risk assessment. In this context, TCR gene defined mutant set expansion is a confounding observation because it negates the assumed equivalence of mutant frequency with mutation frequency. It will be necessary to differentiate between these two possibilities in interpreting the significance of elevated mutant frequency values in humans. We have made progress in developing an alternative to the characterization of isolated mutant colonies for estimations of the contribution of TCR gene defined mutant sets to the total *hprt* mutant T-cell population in an individual. The method is based on the premise that the distribution of individual TCR gene defined mutant clones, as analyzed in isolated colonies, would be maintained in a mass culture of TG^r cells. A low frequency of TCR gene defined mutant sets as in individuals D, E, and F in Table 1 does not really affect the assumption that mutation frequency is equivalent to mutant frequency. Therefore, a method

that screens for the absence of TCR gene defined sets would be useful even if it did not allow a quantitative measure of the magnitude of this fraction if present, that is a + or - assay. If such a method showed the presence of TCR gene defined sets, further study could be pursued to analyze the magnitude and significance of the clonal population.

For our initial studies, we have employed cell samples from patients with multiple sclerosis (MS) because of the evidence that such individuals contain larger populations of dividing T-cells than normal individuals (Allegretta et al., abstract). If cell proliferation increases the probability of mutation, those individuals with elevated mutant frequencies would be likely candidates for the presence of TCR gene defined mutant sets. Elevated *hprt* T-cell mutant frequencies have been noted in patients with chronic progressive MS (Sriram et al., 1989). Therefore, mass cultures of T-cells were grown to cell populations of approximately 10 x 10^6 cells in the absence or presence of 10^{-5}M TG. Southern blot analysis was performed on HindIII digests using the TCR β gene probe. For a mass culture, the expected result is the appearance of only TCR gene germ line fragments on such blots because each unique TCR gene rearrangement fragment is present in too low a fraction of cells to result in visible bands. Bands representing TCR gene germ line fragments are still visible because most clones retain one or more TCR gene germ line conformations. However, if there is a TCR gene defined set of cells which predominates in the mass culture, its uniquely rearranged TCR gene pattern should be visible on Southern blots.

Preliminary results of this sort of analysis are shown in Figure 3. Cultures from six individuals are shown in pairs of lanes. The left lane of each pair (lanes 1, 3, 5, 7, 9, and 11) represent DNA from mass cultures grown in the absence of TG and the right lane (lanes 2, 4, 6, 8, 10, and 12), mass cultures grown in the presence of 10^{-5}M TG. Lanes 9 and 10 serve as a positive control for the method as these lanes contain cultures from individual G (Table 2: 95% TCR defined sibling mutants). Lane 9 shows the germ line TCR β gene pattern of three bands of 14.0, 8.0, and 3.5 kb (the V, $C_\beta 2$ and $C_\beta 1$ regions of the TCR β gene, respectively) as expected, while lane 10 shows the TCR gene defined mutant set's TCR β gene rearrangement pattern that was found in the individually analyzed mutant colonies from individual G, as expected. Lanes 11 and 12 contain DNA from unselected and TG selected mass cultures, respectively, from a normal individual with low *hprt* T-cell mutant frequency. Both lanes show the germ line pattern, consistent with the absence of a TCR gene defined set in the TG^r mutant population. In the samples from MS patients, the unselected mass cultures show the germ line pattern (lanes 1, 3, 5, and 7) while 3 of 4 TG^r selected cultures show a rearranged pattern (lanes 2, 6, and 8). Two samples show the germ line bands plus 2 new bands (lanes 2 and 6) while the third shows only a rearranged pattern (lane 8). Confirmation of the presence or absence of TCR defined sibling mutant colonies in these 4 MS patients requires the analyses of individual mutant colonies and is in progress. However, these results do demonstrate the feasibility of employing this mass culture approach to screen for TCR gene defined mutant sets in an individual's T-cell population. This can be employed as a pretest for the study of TCR gene defined mutant sets <u>per se</u>, as with the MS patients, or as a post-test for the small number of blood samples that show elevated mutant frequencies when the T-cell assay (autoradiographic or cloning) is used for human population monitoring. The latter use will aid in judging the significance of an elevated frequency for an individual, and constitutes a

significant advance in the use of T-lymphocytes for *in vivo* human mutagenicity studies.

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Table 1

Use of TCR gene rearrangement patterns
to define mutation frequency in normal adults

	Individual		
	D	E	F
# of mutants	90	141	95
# of different TCR patterns	82	120	85
% unique	91%	85%	89%
Mean mutant frequency	4.8×10^{-6}	4.7×10^{-6}	5.6×10^{-6}
Mean mutation frequency	4.4×10^{-6}	4.0×10^{-6}	5.0×10^{-6}
# of TCR gene sets	5 doublets 1 quadruplet	9 doublets 1 quadruplet 1 nonamer	3 doublets 2 triplets 1 quadruplet
fraction of mutants with *hprt* structural alterations	13/90=0.14	23/141=0.16	12/95=0.13
fraction of mutations with *hprt* structural alterations	13/82=0.16	20/120=0.17	8/85=0.09

Table 2

Use of TCR gene rearrangement patterns to define mutation frequency in "outlier" adults

	Individual G	H	I
# of mutants	31	32	13
# of different TCR gene patterns	2	20	9
% unique TCR gene patterns	6%	63%	69%
hprt mutant frequency	470×10^{-6}	69×10^{-6}	52×10^{-6}
"conservative" *hprt* mutation frequency	28×10^{-6}	43×10^{-6}	36×10^{-6}
hprt mutant isolates with a # of identical TCR gene patterns	30	13	5

Table 3

Distribution of deletion breakpoints in *hprt* mutations

Group	5′ flanking	Intron number (size in kb) 1 (13.8 kb)	2+3 (15.0 kb)	4-8 (13.2 kb)	3′ flanking
A adults (31 mutations)					
number	13	9	9	7	17
percent	24	16	16	13	31
B newborns (22 mutations)					
number	5	15	17	1	6
percent	11	34	39	2	14

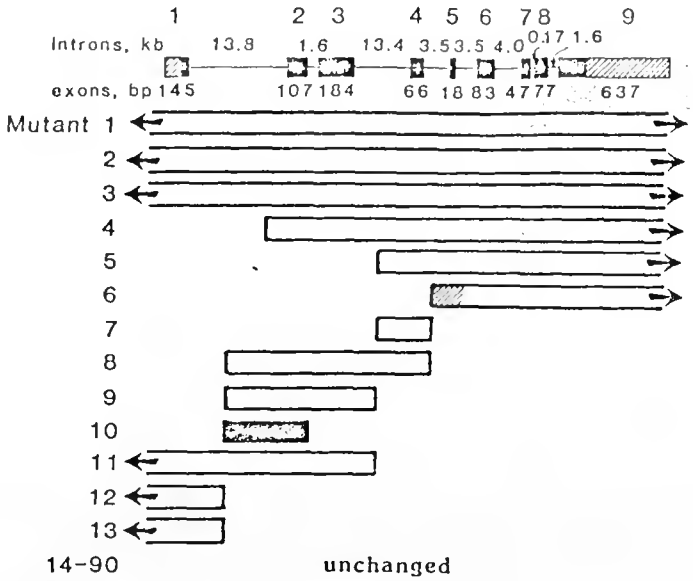
FIGURE LEGENDS

Figure 1.
Deletion and rearrangements found in TG^r mutant colonies from 3 individuals. The boxed areas represent the *hprt* exons deleted as analyzed by Southern blots of PstI and HindIII digests. Solid boxed areas indicate regions of complex rearrangements. Shaded areas indicate regions which may or may not be deleted. Arrows indicate deletions extending beyond the *hprt* sequences an unknown distance. Superscripts: 1 indicates 4 TCR sibling mutants from individual E; 2 indicates 2 different TCR sibling mutants from E; 3 indicates 4 TCR sibling mutants from F; 4 indicates 2 different TCR sibling mutants from F with the same TCR β but different TCR γ patterns.

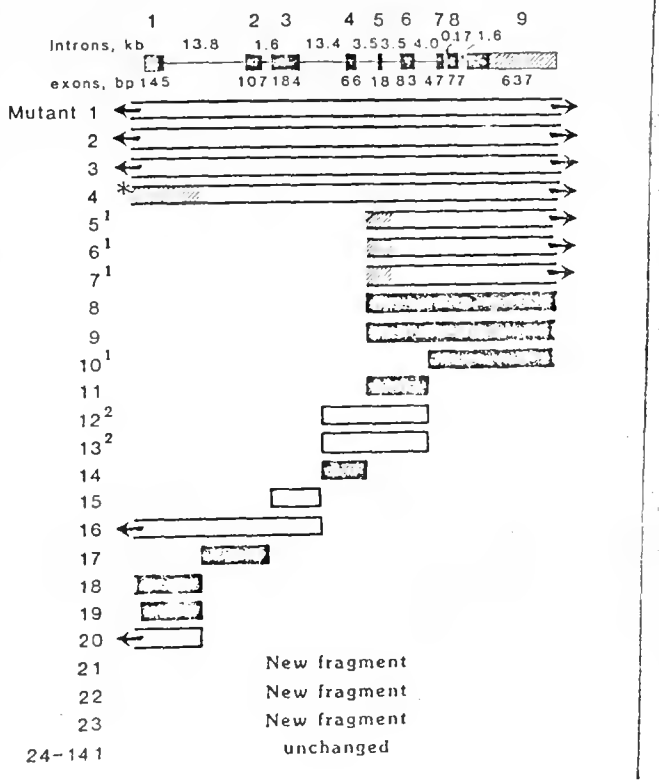
Figure 2.
Southern blot analyses with X-chromosome probes DXS10 and DXS144. Southern blots of PstI digests of 4 wild type (lanes 1-4 numbered from left to right) 5 total *hprt* deletions (lanes 5-9) and 3 partial *hprt* deletions (lane 10, exon 1 deletion; lane 11, exons 5-9 deletion; and lane 12, exons 4-9 deletion) were hybridized with the DXS10 (top panel) or DXS144 (bottom panel) probes. The total deletion mutants in lanes 6 and 8 show deletion of the DXS10 sequences but not the DXS144. The other 10 clones retain both sequences.

Figure 3.
Southern blot analyses with the TCR β gene probe. Mass cultures of T-lymphocytes were analyzed by Southern blotting of HindIII digests with a TCR β gene probe. The mass cultures were from 4 different multiple sclerosis patients (lanes 1 and 2, 3 and 4, 5 and 6, and 7 and 8 respectively). Individual G (Table 2) who shows 95% TCR defined sibling mutants is the + control for TCR sibling mutants (lanes 9 and 10) and a normal individual with a mutant frequency of 5-8 x 10^{-6} is the negative control (lanes 11 and 12). The mass cultures were grown in the absence (odd numbered lanes) or presence (even numbered lanes) of 10^{-5} M TG.

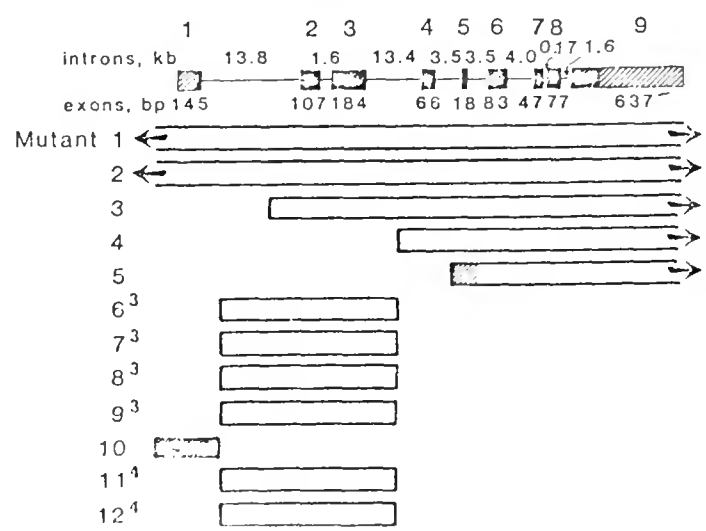
Individual D



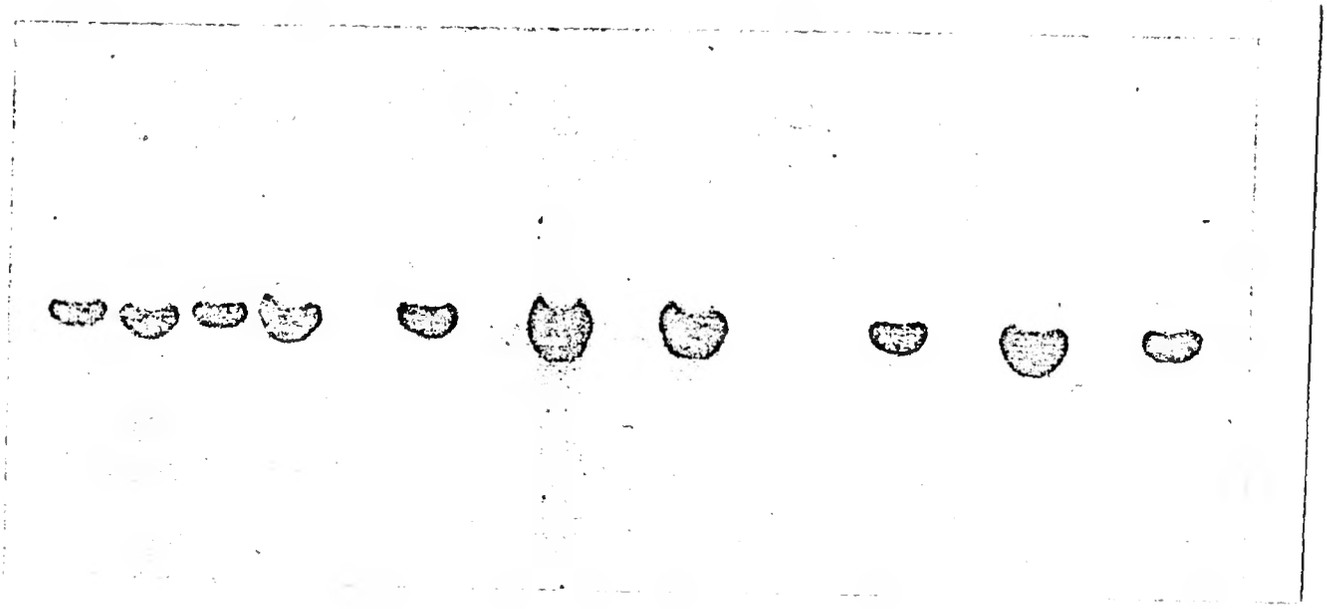
Individual E



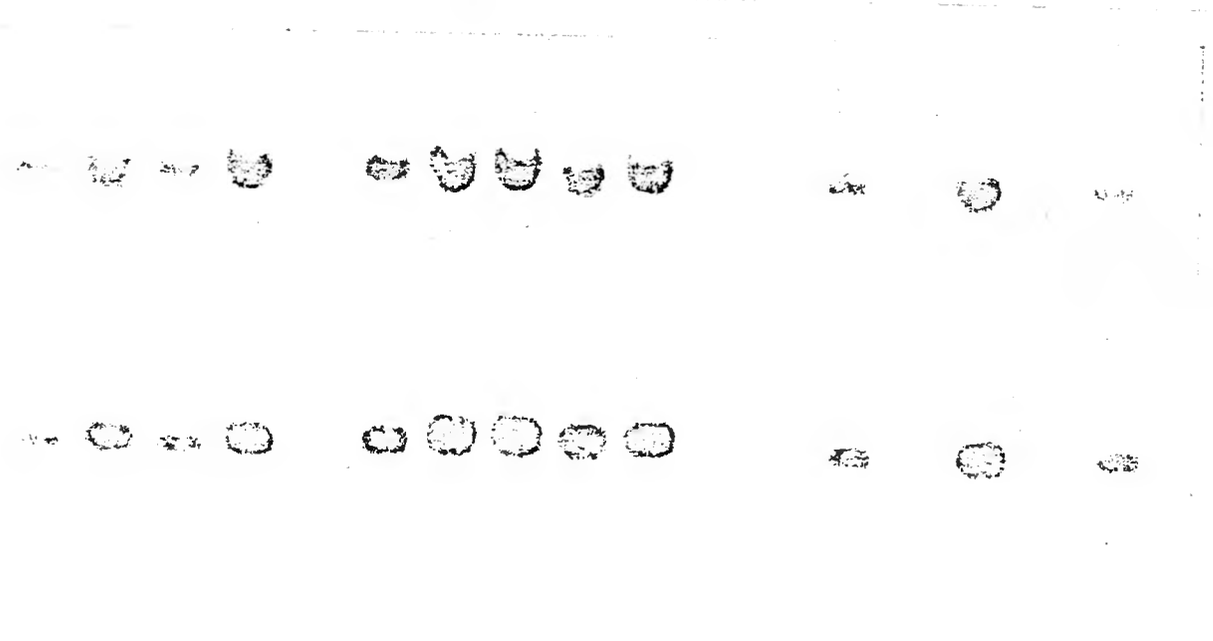
Individual F



A) PROBE DXS10



B) PROBE DXS144



LANE	1	2	3	4	5	6	7	8	9	10	11	12
										EXON1	EXONS5-9	EXONS4-9
	WILD TYPE				TOTAL DELETIONS					PARTIAL GENE DELETIONS		

TCRβ-HIND III