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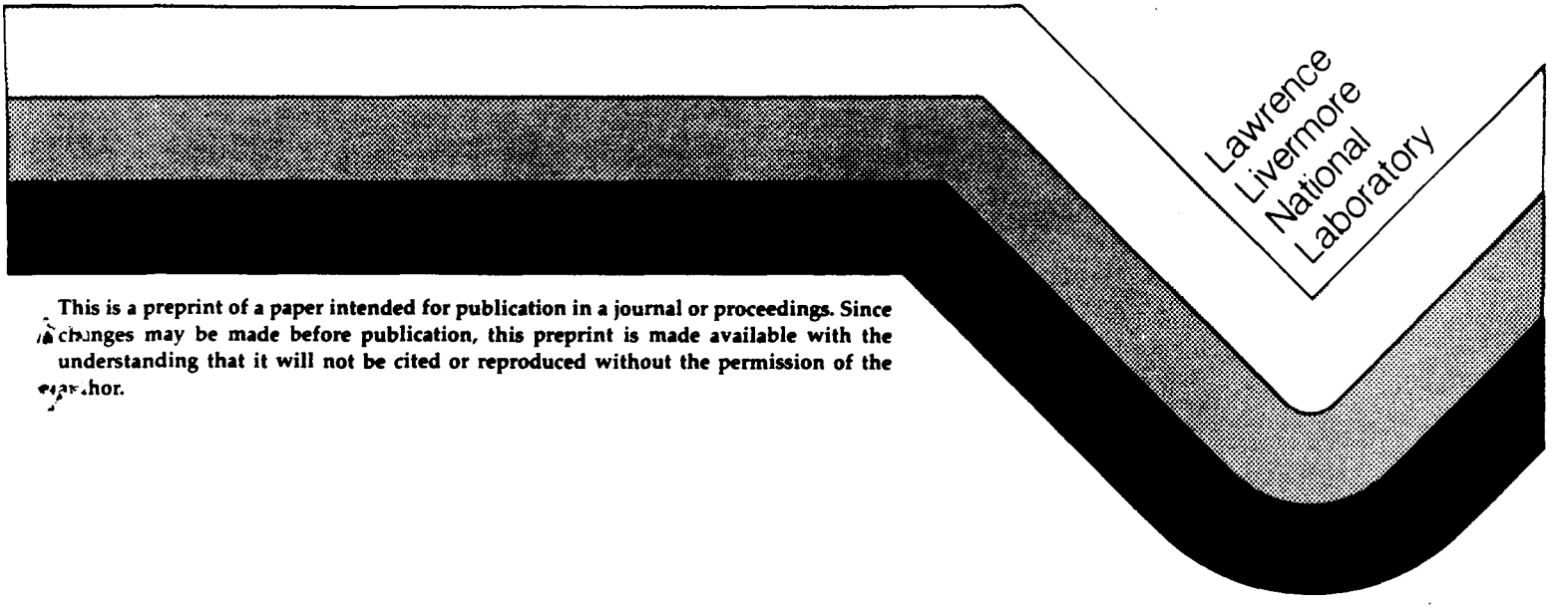

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MOUSE MODEL FOR SOMATIC MUTATION AT THE HPRT GENE: MOLECULAR AND CELLULAR ANALYSES

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INTRODUCTION

Our goal is to use the mouse to model the organismal, cellular and molecular factors that affect somatic mutagenesis in vivo. A fundamental tenet of genetic toxicology is that the principles of mutagenesis identified in one system can be used to predict the principles of mutagenesis in another system. The validity of this tenet depends upon the comparability of the systems involved. To begin to achieve an understanding of somatic mutagenesis in vivo, we have been studying mutations that occur in the hypoxanthine phosphoribosyltransferase (HPRT) gene of lymphocytes of mice.

Our in vivo model for somatic mutation allows us to analyse factors that affect somatic mutation. Having chosen the mouse, we are working with cells in which the karyotype is normal, and metabolic and DNA repair capacity are defined by the mouse strain chosen. *At the organismal level*, we can vary sex, age, the exposure history, and the tissue source of cells analysed. (All studies reported here have, however, used male mice.) *At the cellular level*, T lymphocytes and their precursors are the targets and reporters of mutation. Both mature, splenic T lymphocytes and mature thymic cells can be propagated in vitro for selection of mutants, determination of mutant frequency, and isolation of cells for molecular analysis. Concanavalin A is used as the mitogen and lymphocyte growth factors are used to maintain cell proliferation. Our methods (Jones et al., 1985, 1987a) are parallel to those developed by the groups of Albertini

(Albertini et al., 1982; O'Neill et al., 1987) and Morley (Morley et al., 1983, 1985), and adopted by others (as by Messing and Bradley, 1985; Henderson et al., 1986; Hakoda et al., 1988) for analysis of somatic mutation in human lymphocytes. At *the molecular level,* the X-linked HPRT gene is the reporter of mutation. Mutants are recovered as HPRT deficient cells by selection in vitro with the cytotoxic purine analogue thioguanine. Forward mutations to the selectable phenotype require only a single mutation due to the hemizygosity of this gene, real in the male and functional in the female. The background mutation frequency of HPRT deficient cells, $\sim 2 \times 10^{-6}$, makes our system sensitive to induction of elevated mutant frequencies. HPRT deficiency has been associated with both gross alterations and point mutations of the gene in many studies, including heritable mutations of HPRT in man (Yang et al, 1984; Gibbs et al., 1989), somatic mutations in man (Turner et al., 1985; Nicklas et al., 1987) and in the mouse (Jones et. al., 1987b). The size and structure of the murine and human HPRT genes are similar. The murine HPRT gene has 9 exons in 34kb (Melton et al., 1984), while the human HPRT gene has 9 exons in 44kb (Patel et al., 1984). The coding sequences and amino acid sequences for the 2 gene's products are also heavily conserved.

In vivo somatic mutagenesis involves complexities not found in in vitro studies. Whereas in vitro studies employ cells in a closed system and last at most a few weeks, studies of lymphocytes in vivo focus on a hematopoietic system in which new cells may be produced from undifferentiated precursors as well as from preexisting mature cells over the life span of the individual, 2-3 years for a mouse. The relative contribution of each source to the pools of mature lymphocytes, and to pools of long lived lymphocytes, depends upon age, immunologic history, and exposure history of the individual. As a result, the identity and characteristics of cells that are targets of mutation, and their relationship to the recovered mutant cells, depend upon these factors as well. Our studies of mutant thymic and splenic T lymphocytes as a function of time and exposure history have elucidated some of the complexities of studying mutagenesis in the hematopoietic system in vivo.

We have used two mutagens, ethylnitrosourea (ENU) and gamma radiation, to explore our in vivo model of somatic mutagenesis. These agents have served as examples of direct acting nonclastogens and clastogens, respectively. Gamma radiation has provided information about the effect of cytotoxicity on the formation and persistence of induced mutations in this model system. ENU, by virtue of the persistence of some of its associated adducts (Den Engelse et al., 1987), has helped us identify some of the factors affecting delay between exposure and conversion of adducts to mutations.

The results we present summarize how studies with these model mutagens have contributed to the understanding of in vivo mutation of the HPRT gene at the organismal, cellular and molecular levels. They also demonstrate the ways in which this mouse model can be used to screen for in vivo mutagenesis and to study basic mechanisms of mutation.

RESULTS

The frequency of mutants induced by gamma radiation has been found to depend upon the tissue studied, the time elapsed since exposure, and the dose/dose rate of exposure. Irradiations were performed with a Cesium-137 source at dose rates of 30-70cGy/min, either as acute or fractionated doses. Comparisons of mutant frequency in the spleen and thymus at weeks 1, 2 and 3 after exposure to doses up to 400cGy revealed a *3 week expression time for spleen cells*, but only a 1 week expression time for thymus cells (Jones et al., 1988). The higher mitotic activity of the thymus probably leads to faster dilution of normal enzyme after the formation of a mutant HPRT gene. Expression time is a fundamental characteristic to be kept in mind when monitoring for genetic effects after an exposure.

The period between exposure and maximum radiation-induced mutant frequency in the spleen was dose dependent. A maximal mutant frequency was observed 3-5 weeks after doses above 100cGy, and 10 weeks after doses below 100cGy (Jones, unpublished results). The highest frequency detected in young adult mice was 220×10^{-6}, 3 weeks after 400cGy. At this time the response of mutant frequency to

dose was curvilinear. Overall, the quadratic component of the dose response was reduced at 10 weeks and later. Mutant frequencies were elevated, but variable and submaximal, at 15, 30 and 53 weeks after exposure to doses of 89 rad or more. Persistence of mutants was not permanent,however; mice exposed to 350cGy had background spleen mutant frequencies 65 weeks (15 months) after exposure. The dependence of the radiation-induced mutant frequency on dose, and elapsed time after dose, may be related to the molecular nature of the mutations induced at different doses as well as alterations of lymphocyte population dynamics in mice subjected to varied levels of cell kill.

We have developed a method to screen for certain classes of deletions of the HPRT gene for our initial studies of the mechanisms of radiation-induced mutation and the consequences of radiation exposure of lymphocytes in vivo. We have used the polymerase chain reaction (PCR) (Saiki et al., 1988) to screen for the retention of exons 1 and 9 in genomic DNA. Deletion of both exons is putative evidence of total deletion of the 34kb gene, whereas deletion of only exon 1 or 9 is evidence of a deletion affecting one end of the gene. By choosing oligonucleotide primers that flank an exon, it is possible to enzymatically amplify exon-specific fragments of DNA (Fig.1). The identity of these products has been confirmed

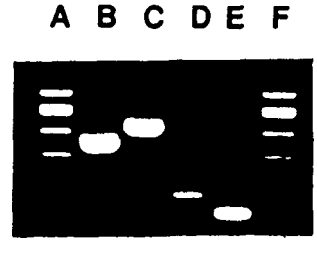

Fig. 1. PCR amplification of individual exons of the murine HPRT gene from genomic DNA of T lymphocytes. Exon specific products of PCR reactions are visualized here by ethidium bromide staining of an agarose gel. Lanes A and F, ϕX174 Hae III size markers. Lanes B, C, D, E: PCR products of exons 1, 3, 8 and 9, respectively.

by sequence analysis (detailed discussion of these methods and results will be published separately).

Analysis of the molecular nature of mutations present in radiation-exposed mice at early and late times

after exposure has provided both expected and surprising results. We have compared the mutations present in spleen cells of mice exposed to gamma radiation 1 month and 15 months after exposure to 400 or 350cGy, respectively, with those present in unexposed controls (Table 1). One month after exposure there was a dramatic increase in the fre-

Table 1. The proportions of complete and terminal deletions were determined 1 and 15 months after irradiation of 3 month old mice.

Exposure History	Age at clone isolation	Exon 1: + / 9: +	Status + / -	- / +	- / -	
None	3 months	13	3	3	7	*
400cGy	4 months	9	1	2	18	#
None	18 months	9	1	4	6	#
344cGy	18 months	10	0	1	3	#

* Southern analysis (Jones et al., 1987b)
\# PCR analysis for retention of exon 1 and 9

quency and proportion of mutants with putative total gene deletions. Mutant frequency at time of isolation was 22-220x10^{-6}, depending upon the individual mouse, hence 10 fold to 100 fold above background. The proportions of deletions we have detected are similar to those reported for radiation-induced mutation in vitro for the Chinese hamster HPRT gene (Vrieling et al. (1985);Breimer et al.(1986);Fuscoe et al. (1986); Thacker (1986)). Surprisingly, mutants isolated 15 months after exposure to 350cGy had a lower proportion of deletions than unexposed, age matched controls. The mutant frequencies (2-3x10^{-6}) of the 2 groups of older mice were indistinguishable from one another and from young adult mice. All mutants had <5% normal hypoxanthine incorporation capacity. The apparent preferential loss of deletion mutations that affect either the whole locus or its termini in mice aged 15 months after radiation suggests that lymphocytes with these deletions are at a selective disadvantage in mice with a history of exposure to a cytotoxic agent. The selection would appear to be due to effects on flanking genetic loci, rather than to deficiency of HPRT. Extension of these studies to larger groups of mutants and more

complete analysis of the mutations will be of considerable interest.

Fractionation of radiation dose led to induction of fewer mutants. At 3 weeks after the last of 8 daily doses of 50cGy, the mutant frequency was 1/6 that at 3 weeks after exposure to a single dose of 400cGy. This observation suggests that repair is more efficient after the 50cGy dose fraction and/or that additional mechanisms of mutation are operative at the higher doses. Analysis of the molecular nature of mutations induced by fractionated doses of gamma radiation will help resolve these possibilities.

Studies with ENU as the mutagen have provided additional insights into unique aspects of in vivo mutagenesis of lymphocytes (Jones et al, 1987a). In contrast to gamma radiation, higher frequencies of mutants were detected in the thymus than in the spleen during the first 3 weeks after exposure to doses up to 58mg/kg, and mutant frequencies in the spleen increased throughout the first 10 weeks after exposure; maximum mutant frequencies in the spleen were ~200×10^{-6}, 10 weeks after 58mg ENU/kg. These features of ENU mutagenesis are probably reflections of the requirement for DNA synthesis to convert adducts to mutations, and the long lived nature of some lymphocytes and certain ENU adducts (Singer, 1986; Den Engelse et al., 1987). Long lived adducts would also explain our observation that fractionation of exposure to ENU (58mg/kg divided into 5 weekly doses) did not alter the frequency of mutants detected. An insight into lymphocyte biology was gained by studying the ENU-induced mutant frequency in the thymus 5 weeks after exposure and later. The thymus mutant frequency was unpredictable, a behavior that probably reflected continuous repopulation of the thymus by small numbers of bone marrow precursors that stochastically sampled the total precursor pool. Another organismal factor, age at time of exposure, reduced the rate of appearance of ENU-induced mutants in the spleen. The age effect was consistent with slow conversion of ENU adducts to mutations in cycling, long lived mature lymphocytes and an age associated decrease in cell production by the thymus.

CONCLUSIONS

Our studies of mutation in mouse lymphocytes demonstrate the power of an experimental model system to analyse factors that affect somatic mutation in vivo. The greatest contribution of the model system will be in studies of exposed individuals, because exposure conditions can be tightly controlled, and relatively large numbers of individuals can be studied. Model systems which have the added virtue of ability to do longitudinal sampling of individuals (see Zimmer et al., this volume) will provide valuable, additional perspectives on the factors that affect somatic mutagenesis in vivo. With sufficient understanding of the organismal and cellular factors involved, it should be possible to model most aspects of in vivo mutagenesis. Determination of the degree of homology of mutational mechanisms at the molecular level will depend upon detailed molecular characterization of many mutations in each species studied.

When using the mouse model to study mutagenesis, one should keep several factors mind. Due to the expression time required for mutation, analyses of mutation will be optimal at a different time from that which is optimal for other endpoints, such as sister chromatid exchange (e.g. Jones et al., 1985) or unstable chromosome aberrations (Jones et al., 1988 versus Kligerman et al., 1989). Our studies suggest that for noncytotoxic exposures the thymus can be used for simple screening of mutagenicity in mice when analysis is performed 1 or 2 weeks after exposure. For cytotoxic as well as noncytotoxic exposures, the spleen is the preferred source of cells. Mutant frequency analyses should be performed at 2 times, for example at 3 and 10 weeks after exposure, to determine whether there are persistent lesions that are converted to mutations. By comparing the frequencies of mutant thymus and spleen cells of mice exposed to noncytotoxic agents, at 2 and 3 weeks after exposure, respectively, one can get an indication of the role of DNA synthesis in formation of induced mutations.

One should analyse both mutant frequency and mutation spectrum when studying in vivo somatic mutagenesis. In the case of radiation exposure discussed here, initially the mutant frequency was increased and

the mutation spectrum was shifted to more total gene deletions. Many months after exposure, when the mutant frequency appeared to have returned to background, the mutation spectrum was shifted to fewer deletions than expected based upon age adjusted controls. Although as yet the numbers of mutants studied are small, these results suggest that mutant frequency is not a trustworthy measure of the likelihood of an altered mutation spectrum.

Future studies of somatic mutation in the mouse will proceed in many directions. Detailed molecular analyses of mutations induced by a variety of agents will contribute to understanding of the effect that genetic target and cellular and organismal factors have on the mechanisms of induction and the detection of somatic mutations. As somatic mutagenesis is studied in strains of mice having different metabolic and DNA repair capacities, using both endogenous genes such as *hprt* and introduced genetic targets, insight into the many variables that affect the mechanisms of induction and the significance of somatic mutations will be greatly enhanced.

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