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Through a Glass, Darkly:[1] Reflections of Mutation From *lacI* Transgenic Mice

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ABSTRACT

The study of mutational frequency (Mf) and specificity in aging Big Blue *lacI* transgenic mice provides a unique opportunity to determine mutation rates (MR) *in vivo* in different tissues. We found that MR are not static, but rather, vary with the age or developmental stage of the tissue. Although Mf increase more rapidly early in life, MR are actually lower in younger animals than in older animals. For example, we estimate that the changes in Mf are 4.9×10^{-8} and 1.1×10^{-8} mutations/base pair/month in the livers of younger mice (<1.5 months old) and older mice (≥1.5 months old), respectively (a 4-fold decrease), and that the MR are 3.9×10^{-9} and 1.3×10^{-7} mutations/base pair/cell division, respectively (∼30-fold increase). These data also permit an estimate of the MR of GC → AT transitions occurring at 5′-CpG-3′ (CpG) dinucleotide sequences. Subsequently, the contribution of these transitions to age-related demethylation of genomic DNA can be evaluated. Finally, to better understand the origin of observed Mf, we consider the contribution of various factors, including DNA damage and repair, by constructing a descriptive mutational model. We then apply this model to estimate the efficiency of repair of deaminated 5-methylcytosine nucleosides occurring at CpG dinucleotide sequences, as well as the influence of the *Msh2*$^{-/-}$ DNA repair defect on overall DNA repair efficiency in Big Blue mice. We conclude that even slight changes in DNA repair efficiency could lead to significant increases in mutation frequencies, potentially contributing significantly to human pathogenesis, including cancer.

THE use of transgenic rodents has greatly facilitated *in vivo* studies of the mechanisms of mutation, DNA repair, and carcinogenesis (Kohler *et al.* 1991; Mirsalis *et al.* 1994; Mirsalis 1995; de Boer and Glickman 1998). While transgenic rodent mutagenicity assays provide a practical approach to the study of genotoxicity, they offer the additional advantage of providing novel insights into mechanisms of mutation. These observations often include unexplained or unexpected responses that reflect the true biological complexity inherent in mammalian systems and challenge our current understanding of these systems. Some recent examples of unpredicted results arising from transgenic rodent mutational assays include the following: (1) an apparent lack of correlation of 2-amino-1-methyl-6-phenylimidazo [4,5-*b*]pyridine- or 2-amino-3,4-dimethylimidazo[4,5-*f*] quinoline-induced tissue adduct levels and mutagenicity with carcinogenicity in target tissues (Okonogi *et al.* 1997; Ochiai *et al.* 1998); (2) a higher-than-expected spontaneous mutation frequency (Mf) in *mrkII* transgenic mice encoding a *lacI* gene with reduced 5′-CpG-3′ (CpG) content, due to increased frequency of GC → AT transition mutations at the few remaining CpG sequences (Skopek *et al.* 1998); (3) a decline in *lacI* Mf during spermatogenesis in younger but not older mice (Walter *et al.* 1998); and (4) chemoprotection by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin against aflatoxin B_1-induced mutation in female but not male *lacI* transgenic rats (A. S. Thornton-Glickman, Y. Oda, G. R. Stuart, J. Holcroft, J. G. de Boer and B. W. Glickman, unpublished results).

Mutations accumulate in a tissue-specific manner during the life span of an organism, contributing significantly to the risk of diseases including cancer. The study of the origin, frequency, and especially the specificity of mutation is a necessary first step toward understanding the fundamental molecular mechanisms responsible for mutation. We recently reported the changes in spontaneous Mf and mutational spectra (MS) with age in the *lacI* transgene recovered from liver, bladder, and brain of Big Blue mice (Stuart *et al.* 2000). Those data enabled us to combine Mf data from aging mice with estimates of cellular turnover to calculate here, for the first time, mutation rates (MR) in young and adult animals. The data show that MR are not static, as might be inferred on the basis of numerous literature reports that quote a single value but, rather, appear to vary as a function of developmental age or level of proliferative activity of the tissue.

The validity of these estimates of MR is strengthened by the use of DNA sequencing to correct for nonindependent mutational events (*i.e.*, clonal expansions) and

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[1] *For now we see through a glass, darkly; but then face to face: now I know in part; but then shall I know even as also I am known.* 1 Corinthians 13:12.

the use of the well-characterized *lacI* transgene as the mutational target. DNA sequence analysis from this laboratory of nearly 20,000 *lacI* mutants recovered from Big Blue rodents indicates that ~410 of the 1080 (38%) nucleotides that encode the *lacI* gene may be recovered as mutations (http://eden.ceh.uvic.ca/sites.htm; B. W. Glickman and J. G. de Boer, unpublished data). These data enable the calculation of Mf per mutable nucleotide.

MATERIALS AND METHODS

Experimental data: Please refer to Stuart *et al.* (2000) for detailed descriptions of the experimental treatments and sources of the Mf data. For convenience, the relevant Mf for liver and brain are provided in a footnote to Table 1.

Definitions: Mutation frequency is defined as the prevalence of mutation in a gene at a specified age, corrected by DNA sequence analysis for possible nonindependent mutational events due to clonal expansion (de Boer *et al.* 1996, 1997). [Note that uncorrected Mf data provide a *mutant* frequency (MF).] Mutation rates (MR) are best described as the change in Mf (ΔMf) per cell division, although at times (*e.g.*, for convenience of comparison to literature values when discussing MR of deaminations of 5-methylcytosine), MR are expressed as the ΔMf per unit time. Accordingly, increases in *lacI* Mf with time are better described as ΔMf rather than MR. A mutational spectrum (MS) describes the nature, nucleotide position, and frequency of mutations that have occurred within a gene (or a defined DNA sequence). Demethylation is used generically to describe the loss or disappearance of 5-methylcytosine (5MC) from mammalian genomic DNA that can occur by spontaneous hydrolytic deamination of 5MC or enzymatically by DNA (cytosine-5)-methyltransferase or DNA demethylase (refer to results and discussion).

RESULTS AND DISCUSSION

Use of mutation frequency rather than mutant frequency: In this article we have chosen to use Mf rather than MF to calculate MR, as the aging data described in Stuart *et al.* (2000; upon which the following discussion is based) reported Mf. Also, Mf may be more representative of the underlying, initial mutational events that contribute to MR, as opposed to MF that include biological processes (such as clonal expansion) that follow the initial mutational event. Finally, it is noted that the corrections from MF to Mf (Stuart *et al.* 2000) were generally rather conservative; the clonal corrections were 9, 20, 14, 17, and 12% in liver and 38, 16, 15, 10, and 11% in brain aged 1.5, 6, 12, 18, and 25 months, respectively. Nevertheless, it is acknowledged that some laboratories prefer that all mutants be considered (*e.g.*, Drost and Lee 1995; Drake *et al.* 1998; Thompson *et al.* 1998; Heddle 1999).

Liver growth and development: Before MR can be calculated, it is necessary to estimate the number of cell divisions (in some cases, more specifically the number of rounds of DNA replication) that have occurred. Two-week-old CBA/C57BL mouse liver contains $\sim 0.8 \times 10^8$ hepatocytes, increasing 1.5-fold to $\sim 1.2 \times 10^8$ cells in 3-month-old mice (Brodsky and Uryvaeva 1985). Noting that the liver of a young adult mouse weighs ~1.5 g, these numbers agree reasonably well with those provided by Buetow (1985), who reported that a 2-month-old male mouse liver contains $\sim 1.16 \times 10^8$ cells/cm^3, with this number decreasing slightly to 0.98×10^8 and 0.88×10^8 cells/cm^3 at ages 12 and 24 months, respectively. Cell proliferation decreases significantly, ~3.3-fold, in the liver of male mice from ages 10–13 weeks (2.5–3.2 months) (Eldridge and Goldsworthy 1996). Although the number of cells in the postnatal liver reach a plateau, DNA synthesis continues at a reduced rate throughout adulthood, resulting in an age-related increase in mean polyploidy (Carriere 1969; Brodsky and Uryvaeva 1977, 1985). Thus, mean DNA ploidy levels in mouse liver double from ages 1 week to 1 month, and thereafter increase steadily, doubling again by 24 months of age (Brodsky and Uryvaeva 1977). This increase in liver polyploidy is accompanied by increase in liver weight but not cell number.

Calculations of MR should therefore consider DNA replication resulting from polyploidization in addition to that contributed by cellular proliferation, since mutations are established (*i.e.*, fixed, as in fixation of mutations) during DNA replication (Stuart *et al.* 2000). From the age of 2–3 weeks, it has been reported that each liver cell in the mouse enters the mitotic cycle from one to six times (three on the average), resulting in an 8- to 10-fold increase in liver mass and about a 3-fold increase in the number of cells. Mature hepatocytes are fully differentiated, self-maintaining cells with low proliferative rate and low, if any, rate of cell elimination from the population during the life of the mouse. The liver cells in newborn mice are diploid but polyploidy levels increase in young animals (Uryvaeva 1981). In adult mice most, if not all, mitoses are polyploid.

The relatively small increases in the postnatal number of liver cells is reflected by the slow growth rate of this tissue. During normal growth, hepatocytes rarely divide—even in young rapidly growing animals, 2–12 days pass between successive mitoses, and several months in adults (Schultze *et al.* 1978; Uryvaeva 1981). Mouse hepatocytes are regarded generally as having a turnover time of 480–620 days (Cameron 1971).

Brain growth and development: In the mouse, proliferative activity associated with brain development appears to be largely completed by 3–4 weeks after birth (Korr 1980). Accordingly, the number of cells in the whole brain of the mouse stabilizes at 0.85×10^8 nuclei (cells) by 1 month of age, with no significant change in this value up to 36 months of age (Franks *et al.* 1974; Buetow 1985). The adult mouse brain is practically mitotically quiescent, except for a small population of glial cells (Korr 1980; Bowman 1985). DNA polyploidy

levels are also known to remain low, mostly diploid, in the adult brain (Winick *et al.* 1972).

Liver mutation frequency and rate: The *lacI* Mf at conception (0.7 months before birth) is zero, since an inherited mutation in any one of the estimated 40 *lacI* transgenes present on mouse chromosome 4 (Dycaico *et al.* 1994) would result in a spontaneous Mf of $\geq 2.5 \times 10^{-2}$, >500-fold higher than the spontaneous Mf of ~ 4–5×10^{-5} normally observed at age 1.5 months (Heddle 1998). Therefore, we were able to calculate that the increase in Mf (ΔMf) during the 2.2-month period from conception (Mf of zero) to age 1.5 months postnatal (4.3×10^{-5}) was 2.0×10^{-5} mut/*lacI* transgene/month. Since ~410 *lacI* nucleotide positions result in mutants recoverable in the Big Blue assay, the ΔMf in animals <1.5 months old was therefore 4.9×10^{-8} mutations/bp/month.

It has been noted previously that spontaneous mutations in somatic cells appear to accumulate steadily throughout adult life (Curtis 1971). This result was confirmed in our mutational studies of Mf in liver of mice that were >1.5 months old (Stuart *et al.* 2000). The least squares plot for the ΔMf in liver of mice >1.5 months old (Figure 1 in Stuart *et al.* 2000) gave a slope of 0.45×10^{-5} mut/*lacI* transgene/month ($R = 0.987$). Dividing by the 410 *lacI* nucleotide positions recoverable as mutants in the Big Blue assay, the ΔMf in animals >1.5 months old was therefore 1.1×10^{-8} mut/bp/month. On the basis of these values, the ΔMf in liver in younger mice (<1.5 months old) increased approximately fourfold faster than in mice >1.5 months old.

We also calculated the MR (the ΔMf per cell division) by using the estimates of cellular proliferation and DNA replication provided above. Since adult liver proliferates slowly but mean polyploidy levels increase, the ΔMf with age in this tissue results primarily from DNA replication in nondividing cells, resulting in fixation of DNA lesions (during translesion bypass) or DNA mispairs as mutations (Stuart *et al.* 2000). Therefore, to facilitate both the calculation of MR in adult liver and to simplify the discussion that follows, polyploidizing DNA replications were considered to be functionally equivalent to cellular divisions.

Since the livers of younger mice, aged 1.5–2 months, contain $\sim 1.3 \times 10^8$ cells, we estimated that ~27 cell divisions (DNA replications) had occurred ($2^{27} = 1.3 \times 10^8$) during the period from conception to age 1.5 months. Dividing the Mf of 4.3×10^{-5} at age 1.5 months by 410 mutable *lacI* nucleotide positions and 27 cell divisions gave an MR of $\sim 3.9 \times 10^{-9}$ mut/bp/division in mice aged up to 1.5 months. The estimate of the number of cell divisions assumed that cell death (for example, due to apoptosis) was negligible. However, it may be noted that the magnitude of this MR estimate is rather modestly affected over a wide range of cell divisions; for example, using half as many (13) and twice as many (54) cell divisions resulted only in an approximately twofold change upward or downward, respectively, in the calculated MR.

We estimated the extent of cellular proliferation (DNA replication) in livers older than 1.5 months as follows. First, the relatively steady increase in Mf (ΔMf) with age in the adult liver suggested that the balance between DNA replication (fixation of DNA mispairs or DNA lesions as mutations) and DNA repair was maintained during this period. Second, Brodsky and Uryvaeva (1977) found that mean polyploidy levels in mouse liver increased only about twofold from ages 1 to 24 months. Third, the turnover time for hepatocytes is 480–600 days (1.3–1.6 years; Cameron 1971), indicating that the population of liver cells is replaced ~1.5 times over 2 years. These observations collectively suggest that from ages 1.5 to 25 months there are probably less than two cell divisions (or DNA replications) on average per cell.

In liver from mice aged 1.5–25 months, by assuming that two cell divisions (DNA replications) had occurred, we calculated the MR to be $\sim 1.3 \times 10^{-7}$ mut/bp/div [= (1.1×10^{-8} mut/bp/month)(23.5 months)/(2 div)]. Assuming that three cell divisions had occurred only reduced the MR 1.5-fold, to 8.6×10^{-8} mut/bp/div.

For convenience, the liver ΔMf and MR values calculated above are summarized in Table 1.

Brain mutation frequency and rate: Since cellular proliferation in the mouse brain is essentially completed by 1 month of age, resulting in 0.85×10^8 cells, it can be estimated that ~27 cell divisions have occurred ($2^{26} = 0.67 \times 10^8$; $2^{27} = 1.3 \times 10^8$). Using this value and the brain Mf at 1.5 months of 2.9×10^{-5}, we calculated that the MR in mouse brain from conception (−0.7 months) to age 1.5 months was 1.1×10^{-6} mut/*lacI*/div, or 2.6×10^{-9} mut/bp/div. This compares with the ΔMf during this period of 3.2×10^{-8} mut/bp/month.

The small but statistically significant increase in brain Mf between ages 1.5 and 6 months (Table 1) is consistent with the known low proliferative capacity of brain tissue. The small increase of brain Mf of 1.6-fold suggests that some DNA replication had occurred; thus, it was assumed that the brain cell population probably underwent less than one doubling during this period. Therefore, by using the Mf at 6 months of 4.6×10^{-5} and assuming one cell division, we calculated that in brain aged 1.5–6 months the ΔMf was 9.2×10^{-9} mut/bp/month, and the MR during this period was $\sim 1.7 \times 10^{-5}$ mut/*lacI*/div (or 4.2×10^{-8} mut/bp/div).

Finally, since there was no significant ΔMf in brain greater than 6 months old, the ΔMf and the MR in brains older than 6 months were practically zero (not detectable). This result is consistent with the fact that adult brain is essentially mitotically quiescent, and thus the contribution to MR from proliferative mechanisms is negligible.

TABLE 1

Summary of mutation frequencies and mutation rates in aging mouse liver and brain

Tissue	Age (mo)	No. of divisions	ΔMf[a] (mut/bp/mo)	MR (mut/bp/div)	Ratio of ΔMf/MR
			All mutations		
Liver	(−0.7)−1.5	27	4.9×10^{-8}	3.9×10^{-9}	13
	1.5–25	2	1.1×10^{-8}	1.3×10^{-7}	0.085
		(Ratio)	(4.5)	(0.03)	
Brain	(−0.7)−1.5	27	3.2×10^{-8}	2.6×10^{-9}	12
	1.5–6	1	9.2×10^{-9}	4.2×10^{-8}	0.22
		(Ratio)	(3.5)	(0.06)	
		6–25	0	0	0
			Deaminations (deam/5MC/mo)	(deam/5MC/div)	
Liver	(−0.7)−1.5	27	6.0×10^{-8}	4.9×10^{-9}	12
	1.5–25	2	2.3×10^{-8}	2.7×10^{-7}	0.085
		(Ratio)	(2.6)	(0.02)	

deam, deaminations (of 5-methylcytosine); div, divisions; 5MC, 5-methylcytosine; mut, mutations.

[a] ΔMf is the change (increase) in Mf during the indicated period of time. The Mf were as follows: liver, 4.3×10^{-5} at 1.5 mo, 14×10^{-5} at 25 mo; brain, 2.9×10^{-5} at 1.5 mo, 4.6×10^{-5} at 6 mo, and 5.0×10^{-5} at 25 mo (taken from Stuart *et al.* 2000).

These brain ΔMf and MR data are also summarized in Table 1.

Distinguishing between Mf and MR: Why should we distinguish between Mf and MR? Most data from mutagenicity assays report Mf (the prevalence of mutations) at a specified point in time, while most studies that discuss mutation with regard to human health or evolution report MR (change in prevalence over time). Therefore, we feel that to better understand data from model systems, including the Big Blue assay, we should also understand the nuances between Mf (the accumulated mutational burden) and MR (the rate of increase in Mf per cell division, or less preferably, unit of time).

According to the data in Table 1, the increase in Mf (ΔMf) in liver of younger mice (<1.5 months old) occurred more than fourfold faster than in older (≥1.5 month) mice. Conversely, the MR in younger mice (<1.5 month) was ~33-fold slower than in older mice (≥1.5 month). The simplest explanation for this seemingly paradoxical relationship between ΔMf and MR is that in developing tissues DNA replication probably contributes more to increases in Mf (*e.g.*, through generation of replication-dependent DNA mismatches) than does DNA damage (nonreplication-dependent premutagenic lesions, *e.g.*, hydrolytic deamination of 5MC). This is supported by the observation that the Mf that had accumulated during the first 2.2 months of life (*i.e.*, from conception to 1.5 months postnatal) required an additional 10.5 months to double in frequency. Thus, in younger animals the ΔMf is greater than in older animals. Conversely, during the period of rapid cellular proliferation in developing mouse liver, the spontaneous mutations that occurred were relatively quickly partitioned among progeny daughter cells, resulting in a lower MR in younger animals.

Another way of describing the changes in ΔMf and MR with age is to note that (1) ΔMf appear to be proportional to cellular proliferation (ΔMf $\propto$ div); (2) MR appear to be inversely proportional to cellular proliferation [MR 1/$\propto$ div; such a relationship was speculated previously by Drost and Lee (1995) in their interesting article on germline MR]; and (3) cellular proliferation varies inversely with age (div 1/$\propto$ age). By substituting expression 3 into expressions 1 and 2, we see that as age increases, ΔMf decreases and MR increases, consistent with observed values.

The data summarized in Table 1 clearly indicate that MR in a tissue are not constant throughout the life span of an animal. Interestingly, however, for comparable developmental periods of growth the values for the ΔMf as well as the MR in liver and brain are remarkably similar. Whether or not this similarity is coincidental remains to be determined.

Deamination of 5-methylcytosine: The most prevalent spontaneous mutation, >25% of all mutations in mammalian tissues, are GC → AT transitions occurring at CpG sequences (Stuart *et al.* 2000). These mutations are generally attributed to hydrolytic deamination of 5-methylcytosine (5MC) bases that are present at CpG sequences (Coulondre *et al.* 1978; Bird 1980; Cooper and Youssoufian 1988; Cooper and Krawczak 1989). The *lacI* gene contains 190 CpG sequences, considering both DNA strands (Farabaugh 1978); of these sequences, 84/190 (44%) have been recovered as mutants in the Big Blue *lacI* assay (B. W. Glickman and J. G. de Boer, unpublished data; http://www.eden.ceh.uvic.ca/

sites.htm). Using the Mf data from our aging study (Stuart *et al.* 2000), we calculated the ΔMf of GC → AT mutations at CpG sequences in liver of mice aged 1.5–25 months to be 0.19×10^{-5} mut/*lacI*/month, corresponding (after dividing by 84 recoverable CpG mutations per *lacI* transgene) to 2.3×10^{-8} deaminations/5MC/month.

The 5MC deamination MR determined in mouse liver was compared to those determined *in vitro* and *in vivo* in double-stranded DNA from different taxonomic groups (Table 2). Several observations are immediately apparent. First, the rate of spontaneous hydrolytic deamination of 5MC measured *in vitro* is amply sufficient to account for the MR observed *in vivo.* The very low *in vivo* deamination rates indicate that repair of GT mispairs must be highly efficient, perhaps >99% (Yang *et al.* 1996). Second, the significant lowering of MR from *Escherichia coli* to mice, apes, and humans, respectively, indicates that MR are not uniform among different taxonomic groups or evolutionary time (Wilson and Jones 1983; Li and Tanimura 1987; Wilson *et al.* 1987; Matsuo *et al.* 1993; Li *et al.* 1996). While these generalizations are not new, the reasonably accurate determination of this rate in transgenic rodents strengthens the validity of these observations.

Caveats associated with the MR determined for deamination of 5MC in the *lacI* transgene include the belief that the bacterially derived *lacI* transgene is fully methylated (Kohler *et al.* 1990; Scrable and Stambrook 1997; de Boer and Glickman 1998; You *et al.* 1998), and is therefore nontranscribed (Provost and Short 1994). The transgenes are stably integrated into the mouse genome as a tandem array of ~40 copies at a single locus on chromosome 4 (Dycaico *et al.* 1994). Also, the density of CpG sequences in the *lacI* transgene is higher than the average density of these sequences in the mammalian genome (de Boer and Glickman 1998). Nevertheless, MR determined in the *lacI* transgene are likely to be reasonably accurate estimates of the average rate occurring throughout the mouse genome for several reasons. First, mutations in the *lacI* transgene are thought to be neutral and therefore confer no selective growth advantage or disadvantage to the cell. Second, the consideration of MR per mutable site (base pair) effectively normalizes the data, with respect to under- or overrepresentation of CpG sequences throughout the genome. Finally, although this conclusion is sometimes debated, it appears that DNA repair activity is not significantly different in the *lacI* transgene compared to endogenous mammalian genes, as similar mutational responses have been observed in the *lacI* transgene compared to the endogenous mouse genes *Dlb-1* (Tao *et al.* 1993) and *Hprt* (Skopek *et al.* 1995; Walker *et al.* 1996). As well, the change in the *lacI* spontaneous mutational spectrum observed in *Msh2*$^{-/-}$ *lacI* cotransgenic mice indicates that *lacI* transgenes respond as predicted to changes in DNA repair function (Andrew *et al.* 1997).

Since Mf in the *lacI* transgene can be ~10-fold higher than those at selected endogenous loci such as *Hprt* (Skopek *et al.* 1995), generalizations on the basis of studies involving the *lacI* transgene are sometimes debated. However, differences in Mf among different genes reflect, in part, differences in the relative sizes of the genes (or perhaps more specifically, the number of nucleotide positions that are recoverable as mutants), as well as the nature of the assay itself. The *lacI* gene is a highly sensitive mutational target, with ≥38% of the nucleotide positions recoverable as mutants, which undoubtedly contributes to elevated Mf. Also, as men-

TABLE 2

Rates of deamination of 5-methylcytosine in double-stranded DNA

Species	Target	MR (deam/5MC/mo)	References
In vitro	M13mp2SV DNA (scored in *E. coli*)	3.9×10^{-5a}	Zhang and Mathews (1994)
In vitro	pSV2-*neo* plasmid reversion assay (scored in *E. coli*)	1.5×10^{-6}	Shen *et al.* (1994)
Mouse	*lacI* transgene (mature liver)	2.3×10^{-8}	This article
Primates	*p53 Alu* sequences (germline)	$\sim 1.4 \times 10^{-9b}$	Yang *et al.* (1996)
Human	Factor IX gene	1.5×10^{-10c}	Koeberl *et al.* (1990)

Deam, deaminations (of 5-methylcytosine); 5MC, 5-methylcytosine; mo, month; MR, mutation rate.

[a] To convert MR cited in the literature as per second to per month, we assumed that 1 mo = 30 days.

[b] This value was estimated by subtracting the MR from humans (1.5×10^{-10} deam/5MC/mo) from the rate constant (1.5×10^{-9} deam/5MC/mo) on the basis of GC → AT transitions from humans and Old World monkeys reported by Yang *et al.* (1996). Alternatively, the average rate constants for the primates provided in Table 1 of Yang *et al.* (1994) are ~(1–2) $\times 10^{-8}$ deam/5MC/year, which is ~(0.83–1.6) $\times 10^{-9}$ deam/5MC/mo.

[c] Koeberl *et al.* (1990) estimated a MR of 3.7×10^{-8} deam/5MC/generation; assuming a generation time of 20 years (suggested by Shen *et al.* 1994), this corresponds to a MR of $\sim 1.5 \times 10^{-10}$ deam/5MC/mo.

tioned, the CpG content of the *lacI* transgene is somewhat higher than in endogenous loci. Nevertheless, since ~40–50% of *lacI* spontaneous mutations involve base substitutions at CpG dinucleotide sequences, these sites likely account for a less than twofold elevation in Mf in the *lacI* transgene relative to endogenous mammalian loci. Finally, we believe that the consideration of Mf (or MR) per base pair may normalize the mutational data, regardless of the locus examined.

Demethylation of DNA: Since demethylation of genomic DNA, principally involving the disappearance of 5MC nucleotides, has been reported to occur at high frequency in aging mammalian cells (Gama-Sosa *et al.* 1983; Wilson and Jones 1983; Hoal-van Helden and van Helden 1989; Mazin 1994), we decided to determine the influence of demethylation on the Mf for deamination of 5MC and vice versa. Demethylation (we use the term generically to describe any disappearance of 5MC and, consequently, the reduction in overall DNA methylation levels) potentially occurs by three routes. (1) Hydrolytic deamination of 5MC can yield thymine directly, resulting in premutagenic GT mispairs (Coulondre *et al.* 1978; Cooper and Krawczak 1989). (2) Immediately after DNA replication, cytosines present in the nascent DNA strands are unmethylated. During the methylation of hemimethylated CpG sequences, DNA (cytosine-5)-methyltransferase covalently bonds at the C6 position of cytosines, greatly labilizing the amino group, resulting in deamination events before methylation at the C5 position (Shen *et al.* 1992; Steinberg and Gorman 1992; Laird and Jaenisch 1996). Thus, cytosines at CpG sequences may be converted directly to thymines, again resulting in premutagenic GT mispairs. These GT mispairs, however they arise, are recognized and repaired with high but not absolute efficiency by thymine DNA glycosylase (Brown and Jiricny 1987; Brooks *et al.* 1996; Marietta *et al.* 1998), resulting in a low but finite accumulation of GC → AT transitions with each round of DNA replication. (3) DNA demethylase, a novel enzyme that specifically recognizes 5MC residues at CpG sequences in mammalian DNA, has been described recently (Bhattacharya *et al.* 1999; Ramchandani *et al.* 1999). The product of this enzymatic reaction is a normal (*i.e.*, nonmutagenic) GC base pair. Since 5MC residues in mammalian genomes occur most frequently at CpG sequences, we can use the mutational data from *lacI* transgenic rodents to evaluate the mechanism principally involved in the demethylation of aging mammalian cells.

Published estimates of the rate of demethylation of mouse and rat genomes vary greatly, even for a single species and tissue such as mouse liver (Gama-Sosa *et al.* 1983; Wilson and Jones 1983; Singhal *et al.* 1987; Wilson *et al.* 1987; Hoal-van Helden and van Helden 1989; Tawa *et al.* 1990; Kanungo and Saran 1992; Mazin 1994, 1995). The reasons for these discrepancies are not obvious but might involve one or more of the following: differences in species, age, strain, and tissues; dietary factors affecting DNA methylation levels; or choice of analytical method (Drahovsky and Boehm 1980; Rein *et al.* 1998). Nevertheless, the estimates of the rate of demethylation are very large; *e.g.*, Wilson *et al.* (1987) determined that the rate of loss of 5MC from the genome of C56BL/6J mouse liver was 0.012% per month. The demethylation rate reported by Mazin (1994) in mice (unspecified tissue) of 0.033% per cell per day (or 0.99%/cell/month) is ~80-fold greater than that reported by Wilson *et al.* (1987). Similar demethylation rates may be inferred from the data described by Hoal-van Helden and van Helden (1989), who reported a 46% decrease in the percentage of 5MC in liver of rats from 1 day before birth to 6 months of age.

Considering the published demethylation data and our MR for GC → AT transitions at CpG sequences, what is the potential influence of demethylation on observed Mf? The mouse genome contains $\sim 7 \times 10^9$ DNA base pairs per diploid nucleus, with ~1 mol% of the cytosines in the liver of C57BL/6 mice methylated as 5MC (Gama-Sosa *et al.* 1983; Tawa *et al.* 1990). [It should be noted that some laboratories report somewhat higher, possibly age-related, values for the 5MC content in young mouse liver. For example, Wilson *et al.* (1987) reported that 3% of the cytosines in 4-wk-old C57BL/6J mouse liver were present as 5MC.] Therefore, assuming that cytosines comprise about one-quarter of the nucleotides in the mouse genome with 1 mol% of these present as 5MC, there are $\sim 1.8 \times 10^7$ 5MC per diploid mouse nucleus. Multiplying this value by the 5MC deamination rate determined in the *lacI* transgene of adult mice (2.3×10^{-8} deam/5MC/month; Table 1) indicates that overall, ~0.4 deaminations of 5MC are expected to occur per month per diploid mouse genome due to spontaneous hydrolytic (and mutagenic) deamination of 5MC.

On the basis of the 5MC content of mouse liver estimated above and the demethylation rate reported by Wilson *et al.* (1987) of 0.012% per month, we estimate that in aging adult mouse liver demethylation results in the disappearance of $\sim 2.2 \times 10^3$ 5MC per diploid nucleus per month, a rate that is $\sim 5.5 \times 10^3$-fold greater than that which occurs due to the deamination of 5MC determined on the basis of data from the *lacI* transgene. That is, if the demethylation rate determined by Wilson *et al.* (1987) was due solely to hydrolytic deamination of 5MC to thymine [or possibly, DNA (cytosine-5)-methyltransferase-mediated conversion of cytosine to thymine during methylation of hemimethylated CpG sequences], the Mf observed in the *lacI* transgene would be $\sim 1.3 \times 10^{-4}$ deam/5MC/month ($= 1.1 \times 10^{-2}$ deam/*lacI* transgene/month), an extraordinarily high ΔMf. On the basis of these calculations, demethylation of genomic DNA at the levels described by Gama-Sosa, Wilson, and others cannot be attributable to spontaneous hydrolytic or enzyme-mediated (mutagenic) deami-

nation events. The recent discovery of a mammalian DNA demethylase specific for 5MC (Bhattacharya *et al.* 1999; Ramchandani *et al.* 1999) therefore provides the most reasonable explanation, at present, for the nonmutagenic hypomethylation of DNA. As mentioned, this enzyme directly converts 5MC to cytosine, resulting in GC base pairs.

A descriptive mutational model: As noted previously (Stuart *et al.* 2000; discussion), mutations occur at different rates in mouse liver and brain tissue, especially in adult tissues. Furthermore, the Mf for the most common spontaneous mutation, deamination of 5MC present at CpG sequences, closely parallels the larger, overall spontaneous Mf. Since these transition mutations are presumably of specific origin, hydrolytic deamination of 5MC, the spontaneous rate of deamination of 5MC *in vitro* is expected (on the basis of thermodynamic considerations, *i.e.*, Arrhenius kinetics) to be constant at a given temperature. Thus, deviations from this rate observed *in vivo* in different tissues must reflect biological influences, including DNA repair.

Observed MR (and Mf) likely reflect a balance between the formation of premutagenic DNA lesions and mispairs, DNA replication (fixation of mutations during translesion bypass, misincorporation at the replication fork), DNA repair, and cell death. Consideration of the effects of these processes on mutation leads to the description of mutation as a function (f),

$$MR_{observed} = f(\text{damage, translesion bypass, misincorporation, repair, death}) \quad (1)$$

that can be expressed as an equation

$$MR_{observed} = (\text{DNA damage rate})(1 - P_{\text{DNA repair}}) \times (P_{\text{mutagenic translesion bypass}})(1 - P_{\text{cell death}}). \quad (2)$$

(It is noted that somewhat analogous models have been proposed by others; *e.g.*, Burkhart and Malling 1993; Drake *et al.* 1998; and Holmquist 1998.) In our model, which may be amended or refined as required, P is the probability of the indicated process. The DNA repair term accounts for the repair of preexisting, premutagenic DNA damage and mispairs. The mutagenic translesion bypass term reflects the contribution of DNA replication past the DNA lesion that results in mutation; if there is no misincorporation or if the lesion blocks replication (which is likely to be a lethal event), then there is no contribution to the mutation rate. The cell death term is included in the event that a mutational treatment results in significant apoptotic or necrotic cell death that might affect the observed MR; under normal circumstances, this effect is believed to be negligible. Finally, it is noted that nucleotides are sometimes misincorporated during replication of nondamaged DNA; however, these mispairs do not immediately contribute to the MR but rather contribute subsequently via the other terms in the equation.

It follows that

$$\Delta Mf_{observed} = (MR_{observed})(\text{elapsed time}). \quad (3)$$

Interestingly, under equilibrium (steady-state) conditions, DNA replication contributes a linear increase to observed Mf according to the formula

$$Mf_i = Mf_{initial} + ((MR)/2)(i), \quad (4)$$

where i is the number of cell divisions (or DNA replications) that have occurred. For example, assuming an initial population of 10^6 largely wild-type cells but containing 100 *lacI* mutants and having a MR of 10^{-5} new mutants per cell division (or DNA replication), after 5 cell divisions the initial Mf of 1.00×10^{-4} will have increased to 1.25×10^{-4}. (The Mf increases are 1.05×10^{-4}, 1.10×10^{-4}, 1.15×10^{-4}, 1.20×10^{-4}, and 1.25×10^{-4} after 1, 2, 3, 4, and 5 divisions, respectively.) Thus, ratios of Mf such as spontaneous *vs.* induced reflect overall differences in MR.

In time, sufficient data might be accumulated for each of the factors inherent in Equation 2 to permit the quantitative evaluation of mutation *a priori.* Nevertheless, in the interim this model might serve as a useful model for evaluating qualitatively or semiquantitatively the relative contributions of various factors to Mf observed *in vivo* or *in vitro.*

To illustrate, we applied our descriptive mutational model to calculate the efficiency of repair of GT mispairs in mouse liver DNA arising due to deamination of 5MC. Since mouse hepatocytes are relatively long lived, and apoptotic indices in adult mouse liver are typically ~0.007% (James *et al.* 1998), we may ignore the cell death term. The translesion bypass term can probably be eliminated since unrepaired GT mispairs will be faithfully replicated, more or less. Therefore, substituting the rate of hydrolytic deamination of 5MC determined *in vitro* (Table 2) and the ΔMf due to GC → AT at CpG sequences (Table 1) into Equation 2

$$2.3 \times 10^{-8}\ \text{deam/5MC/month} = (1.5 \times 10^{-6}\ \text{deam/5MC/month}) \times (1 - P_{\text{DNA repair}})$$

we calculate that DNA repair of GT mispairs in mouse liver was ~98.5–99.9% efficient, depending on the *in vitro* MR chosen. (Note that in this instance, we needed to use the ΔMf value from Table 1 rather than the MR for the units to cancel.) Similarly, we predict that since the human 5MC deamination rate is slower than in mice, the efficiency of repair of GT mispairs is higher, ~99.99–99.9996%, on the basis of the data from Table 2.

In a second application of our mutational model, the recent report of spontaneous mutations in the *lacI* transgene of DNA-repair-proficient and $Msh2^{-/-}$ mice (Andrew *et al.* 1997) provides the opportunity to estimate the contribution of this DNA repair pathway to observed Mf. In the three tissues examined (small intestine, thymus, and brain), MF were elevated ~11-, 15-

and 5-fold, respectively, in $Msh2^{-/-}$ mice compared to control animals. Using the thymus as an example and assuming that the differences in the observed MF in control and $Msh2^{-/-}$ mice were attributable solely to the DNA repair defect, Equation 2 may be simplified as a pair of equations

$$3.1 \times 10^{-5} = (\text{constant})\,(1 - P_{\text{DNA repair in control thymus}})$$

$$47 \times 10^{-5} = (\text{constant})\,(1 - P_{\text{DNA repair in } Msh2 \text{ thymus}})$$

that can be rearranged by substitution of terms to give

$$P_{\text{DNA repair in } Msh2 \text{ thymus}} = (15.16) \times (P_{\text{DNA repair in control thymus}}) - 14.16.$$

Thus, if we make the assumption that DNA repair in the control thymus was 99.99% efficient, then the equation suggests that DNA repair in the $Msh2^{-/-}$ thymus would have been 99.848% efficient, a very small change in overall DNA repair. (Note that under these conditions, DNA repair efficiency in the *Msh2* thymus would apparently drop to zero as overall DNA repair efficiency in the control thymus drops to 93.4%.) Whether or not these conclusions are correct remains to be validated. As well, it cannot be excluded that small differences in the extent of DNA replication or cell death in $Msh2^{-/-}$ mice relative to control animals also contribute significantly to the differences in spontaneous Mf observed in these animals. Nevertheless, the model predicts that subtle differences in the efficiency of DNA repair might profoundly influence observed Mf. If this is true, one implication is that mutator phenotypes (Loeb 1991) could be attributable to small perturbations in the efficiency of DNA repair.

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