Mutation Frequency and Specificity With Age in Liver, Bladder and Brain of *lacI* Transgenic Mice

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ABSTRACT

Mutation frequency and specificity were determined as a function of age in nuclear DNA from liver, bladder, and brain of Big Blue *lacI* transgenic mice aged 1.5–25 months. Mutations accumulated with age in liver and accumulated more rapidly in bladder. In the brain a small initial increase in mutation frequency was observed in young animals; however, no further increase was observed in adult mice. To investigate the origin of mutations, the mutational spectra for each tissue and age were determined. DNA sequence analysis of mutant *lacI* transgenes revealed no significant changes in mutational specificity in any tissue at any age. The spectra of mutations found in aging animals were identical to those in younger animals, suggesting that they originated from a common set of DNA lesions manifested during DNA replication. The data also indicated that there were no significant age-related mutational changes due to oxidative damage, or errors resulting from either changes in the fidelity of DNA polymerase or the efficiency of DNA repair. Hence, no evidence was found to support hypotheses that predict that oxidative damage or accumulation of errors in nuclear DNA contributes significantly to the aging process, at least in these three somatic tissues.

AGING is a complex biological phenomenon, which is reflected by the numerous and diverse theories of aging that have been proposed (Medvedev 1990; Bernstein and Bernstein 1991; Kowald and Kirkwood 1996). Theories of aging involve consideration of various forms of damage to cellular organelles and molecules, including DNA. Many of the nongenetic factors have been collectively considered as a "network theory of aging," integrating the contributions of defective mitochondria, aberrant proteins, and free radicals (Kowald and Kirkwood 1996). Other theories of aging invoke DNA damage as the primary cause of aging (Szilard 1959; Curtis 1971; Gensler and Bernstein 1981). For example, the somatic mutation theory predicts that the frequency of mutations should increase with age (Szilard 1959; Alexander 1967; Morley 1995). Interest in mutational theories of aging reflects the fact that many genetic diseases, like cancer, are more prevalent in older populations.

The study of mutation *in vivo* is facilitated through the use of transgenic rodents, in which mutational responses can be measured in virtually any tissue as a function of age, sex, and diet. The mutational target in Big Blue transgenic mice and rats (Kohler *et al.* 1990, 1991; Provost *et al.* 1993; Dycaico *et al.* 1994) is the exceptionally well-characterized *lacI* gene from *Escherichia coli*. The *lacI* gene is highly sensitive to base substitution and frameshift mutations, as well as small deletions and insertions, making the transgene an ideal choice for recovery of spontaneous and induced mutations (de Boer and Glickman 1998). As well, spontaneous mutational spectra (MS) have been carefully determined for a variety of tissues, providing a reference or baseline for evaluation of age-related or induced mutational effects (de Boer *et al.* 1997, 1998). Studies from our laboratory, and others, have demonstrated that MS are unique for each chemical and physical agent examined (Glickman *et al.* 1995). All mutagens examined to date induce characteristic mutational spectra in the *lacI* transgene. Indeed, significant changes in mutational specificity have been recovered from treated animals despite changes in mutant frequencies (MF) of less than twofold, as for example, with *tris*(2,3-dibromopropyl)phosphate (de Boer *et al.* 1996b) and oxazepam (Shane *et al.* 1999).

In this study the Big Blue mutational assay (Kohler *et al.* 1991; Provost *et al.* 1993) was used to investigate the frequency and specificity of mutation in the *lacI* transgene *in vivo* as a function of age in liver, bladder, and brain of mice. Although the mutation frequencies (M*f*) increased in aging proliferating tissues, there were no significant age-related differences among the various MS in nuclear DNA of mice up to 25 months of age. The absence of age-related changes in the MS in these three diverse tissues does not support a significant role for aging theories that predict that oxidative damage or the accumulation of genetic errors ("error catastro-

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phe") are a major determinant of aging. Additionally, the relatively modest increases in M*f* (approximately threefold in bladder of mice 12 months old and in liver of mice aged 25 months) suggest that the contribution of spontaneous mutations to the aging process is minimal.

MATERIALS AND METHODS

Mice: The animals used in this study were male hemizygous λLIZ/*lacI* (Big Blue) transgenic C57BL/6 mice (Taconic, Germantown, NY). The animals were housed at 20° with a 12-hr light cycle (6 AM to 6 PM). Purina Mouse Chow 5015 (Ralston Purina Company, St. Louis) and water were provided *ad libitum.* The mice were maintained in the University of Victoria Animal Care Unit under standards conforming with the National Institites of Health Guide for the Care and Use of Laboratory Animals. At the appropriate ages, mice were sacrificed by CO_2 asphyxiation followed by cervical dislocation, and tissues were immediately dissected, flash-frozen in liquid nitrogen, and stored at −80°.

Genomic DNA isolation: High molecular weight mouse genomic DNA from liver and brain tissue was isolated using a dialysis purification method (Suri *et al.* 1996). Bladder tissue, which was refractory to disaggregation using Dounce tissue grinders, was minced using a sterile razor blade, immediately digested with proteinase K at 50°, and then dialyzed as previously described.

Big Blue assay: The Big Blue assay was performed following the standardized color-screening assay protocol (Rogers *et al.* 1995; Young *et al.* 1995; Stratagene 1997). To facilitate the identification of *ex vivo* and *in vitro* mutants (Stuart *et al.* 1996), which were excluded from the analysis, generally <16 PFU/cm^2 (≤10,000 plaques per 25 × 25-cm assay tray) were plated.

DNA sequencing and data management: Mutations in *lacI*-bearing λ phage were determined by DNA sequencing using PCR cycle sequencing and automated DNA sequencers, as previously described (Erfle *et al.* 1995). Only *in vivo* (mouse-derived) mutants were considered for analysis (Stuart *et al.* 1996). DNA sequence data were managed and analyzed using custom software (de Boer 1995). To ensure that independent mutational events were analyzed, the data were corrected for possible clonal expansions (de Boer *et al.* 1996a, 1997) by counting only one mutation for those that were recovered more than once from an individual animal. The aging frequency data were corrected accordingly and reported as mutation frequencies (M*f*) rather than uncorrected *mutant* frequencies (MF).

Statistical analyses: Statistical comparisons of MS were made using the Monte Carlo method of Adams and Skopek (Adams and Skopek 1987; Cariello *et al.* 1994) with 2500 iterations, using a program provided by the authors. These tests of significance consisted of pairwise comparisons of MS, using the 12 mutational classes shown in Tables 2–4, as well as the numbers of G:C → A:T and G:C → T:A mutations that occurred at 5′-CpG-3′ (CpG) dinucleotide sequences (Stuart *et al.* 1996). The α-level for significance was set at 0.05. Trends in the mutation frequency data were analyzed using COCHARM (created by Troy Johnson, Procter & Gamble, Cincinnati, OH), a computer program that executes the Generalized Cochran-Armitage test.

RESULTS

Mutation frequency *vs.* age: As shown in Table 1 and Figure 1, there was a statistically significant increase in the M*f* in the *lacI* transgene in liver from mice aged 1.5–18 months. The M*f* at 25 months, although higher, were not significantly greater than those observed at 18 months. M*f* in bladder also increased significantly with age and were significantly higher than those in liver, at all ages examined (1.5, 6, and 12 months). In addition, M*f* in the bladder increased faster than those in the liver. Brain M*f* were lower than those observed either in the liver or bladder, at all ages. Following a small but significant increase in M*f* in mice aged 1.5 to 6 months, no further change was observed in M*f* in adult brain, even at 25 months. "Sectored" mutant frequencies (the frequencies of *in vitro* and *ex vivo* mutants; Stuart *et al.* 1996) are also reported in Table 1; however, these mutants were partitioned from *in vivo* (mouse-derived) mutants and were not sequenced. Sectored mutants are believed to arise due to damaged, unrepaired mouse *lacI* DNA that is subsequently repaired *ex vivo* in *E. coli*; they may also arise *de novo* as the *lacI* λ phage replicates *in vitro.*

Mutational specificity *vs.* age: A subset of the *lacI* mutant λ phage recovered from each tissue and each age group was randomly selected for DNA sequence analysis (Tables 2–4). To facilitate direct comparison of the various spectra by the reader, the data provided in Tables 2–4 are expressed as percentages. For each tissue and all age groups, the predominant class of mutations was G:C → A:T transitions, comprising 34–56% of all mutations, with the majority (62–92%) of these transitions occurring at CpG sequences. The second most common class of mutations was G:C → T:A transversions, which comprised 14–31% of all mutations.

Using the Adams-Skopek (Monte Carlo) algorithm, MS from different age groups from each tissue were compared to determine whether statistically significant changes in MS occurred with age within a tissue type (data not shown). As well, MS from each tissue and age group were compared with each other to determine whether differences existed in the MS among the three tissues. No obvious differences or interpretable trends were observed among any of the mutational spectra.

DISCUSSION

The Big Blue assay provides a versatile and sensitive *in vivo* mutational model. The mutational target in Big Blue mice is the *lacI* transgene, present in a λ shuttle vector that is (stably) integrated as a tandem array of ~40 copies at a single position in chromosome 4 of Big Blue mice (Dycaico *et al.* 1994). It appears that the *lacI* transgene is fully methylated, with cytosines at CpG sequences present as 5-methylcytosine (Kohler *et al.* 1990; de Boer and Glickman 1998; You *et al.* 1998), and is therefore nontranscribed (Provost and Short 1994). Nevertheless, mutational data determined in the *lacI* transgene are likely to be reasonably accurate estimates of those occurring throughout the mouse genome for several reasons. First, mutations in the *lacI*

TABLE 1

Summary of the liver, bladder, and brain mutation frequency data

Tissue	Age (mo)	No. of mice	No. of mutants[a]	Total PFU ($\times 10^6$)	M*f* ($\times 10^{-5}$)	SMF ($\times 10^{-5}$)
Liver	1.5	5	111	2.59	4.29 ± 0.33[b]	2.47
	6	4	135	2.22	6.08 ± 0.55	1.08
	12	4	167	1.99	8.39 ± 1.22	1.25
	18	2	101	0.795	12.7 ± 0.35	6.92
	25	3	114	0.804	14.2 ± 1.13	6.96
Liver totals		18	628	8.40		
Bladder	1.5	5	89	1.58	5.63 ± 0.81	3.41
	6	6	127	1.17	10.8 ± 1.72	9.51
	12	4	142	0.861	16.5 ± 2.22	11.5
Bladder totals		15	358	3.61		
Brain	1.5	5	45	1.56	2.88 ± 0.47	1.92
	6	6	86	1.87	4.60 ± 0.81	3.31
	12	4	62	1.48	4.19 ± 0.56	2.43
	18	2	43	0.874	4.92 ± 0.49	3.09
	25	3	37	0.746	4.96 ± 0.37	2.81
Brain totals		20	273	6.53		

M*f*, mutation frequency; PFU, plaque-forming units; SMF, sectored mutant frequency.
[a] Corrected for possible clonal expansions.
[b] Values represent means ± SE about the mean.

transgene are thought to be neutral and confer no selective growth advantage or disadvantage to the cell. Also, although this conclusion is sometimes debated, it appears that DNA repair activity is not significantly different in the *lacI* transgene compared with endogenous mammalian genes, as similar mutational responses have been observed in the *lacI* transgene compared to the mouse genes *Dlb-1* (Tao *et al.* 1993) and *Hprt* (Skopek *et al.* 1995; Walker *et al.* 1996). Finally, changes in the *lacI* spontaneous mutational spectrum observed in *Msh2*$^{-/-}$ *lacI* cotransgenic mice indicate that *lacI* transgenes respond as predicted to changes in DNA repair function (Andrew *et al.* 1997).

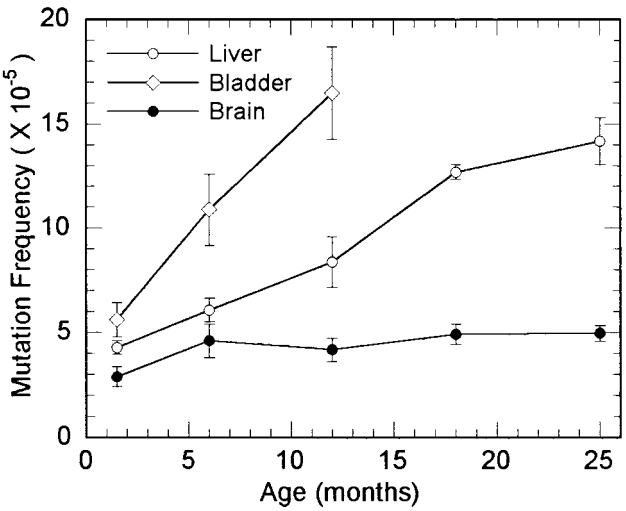

Figure 1.—Mutation frequency *vs.* age in liver, bladder, and brain of Big Blue C57BL/6 *lacI* transgenic mice. Each data point represents the average mutation frequency for that group of animals. The vertical bars indicate the standard error associated with each mutation frequency value.

In this study, we determined M*f* and MS in liver, bladder, and brain of Big Blue mice aged 1.5–25 months. Age-related increases in M*f* are readily detected using standard statistical methods; in this study, trends in M*f* with age were analyzed using the Cochran-Armitage test. Analyses of MS from *lacI* transgenic animals are routinely compared using a computer algorithm described by Adams and Skopek (Adams and Skopek 1987; Cariello *et al.* 1994), a Monte Carlo approximation to Fisher's exact test that is generally regarded (*e.g.*, Piegorsch and Bailer 1994) as one of the most robust methods for statistical comparisons of MS. Therefore, this method was used in this study to evaluate potential changes of MS with age. The application of the Monte Carlo test to analyses of MS is illustrated with selected examples from the literature.

Strong mutagens induce specific mutations at frequencies that result in induced MS that are obviously different from spontaneous MS. For example, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine predominantly induces G:C → T:A transversions and −1 frameshifts in the *lacI* transgene in rat colon (Okonogi *et al.* 1997). Applying the Monte Carlo test to the data provided in Table 3 in Okonogi *et al.* (1997), the MS from 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine-treated and untreated colon were found to be highly significantly different ($P < 10^{-6}$). However, since the spontaneous MS in mice in the present study arose in the absence of strongly mutagenic agents, it is perhaps more relevant to cite examples where significant differences in MS have been detected following treatment with weakly mu-

TABLE 2

Spontaneous *lacI* mutations from liver of Big Blue mice

	1.5 mo			6 mo			12 mo			18 mo			25 mo		
	No.	%	% at CpG	No.	%	% at CpG	No.	%	% at CpG	No.	%	% at CpG	No.	%	% at CpG
Transitions															
G:C → A:T	28	36.4	71.4	37	33.9	67.6	50	33.6	62.0	22	37.3	63.6	32	41.6	62.5
A:T → G:C	3	3.9		13	11.9		16	10.7		5	8.5		1	1.3	
Transversions															
G:C → T:A	23	29.9	65.2	25	22.9	44.0	34	22.8	50.0	13	22.0	38.5	17	22.1	35.3
G:C → C:G	6	7.8	50.0	5	4.6	80.0	8	5.4	37.5	2	3.4	100.0	1	1.3	0.0
A:T → T:A	4	5.2		3	2.8		7	4.7		2	3.4		5	6.5	
A:T → C:G	1	1.3		0	0.0		6	4.0		4	6.8		2	2.6	
Other mutations															
+1 frameshift	2	2.6		6	5.5		6	4.0		1	1.7		0	0.0	
−1 frameshift	3	3.9		13	11.9		8	5.4		2	3.4		4	5.2	
Deletions	4	5.2		3	2.8		5	3.4		1	1.7		7	9.1	
Insertions	0	0.0		2	1.8		3	2.0		0	0.0		1	1.3	
Complex changes	0	0.0		0	0.0		3	2.0		0	0.0		0	0.0	
Double mutants	3	3.9		2	1.8		3	2.0		7	11.9		7	9.1	
Total[a]	77	100		109	100		149	100		59	100		77	100	

[a] The total numbers of mutants after correction for clonality (see materials and methods). The noncorrected mutant totals were 84 at 1.5 mo, 131 at 6 mo, 170 at 12 mo, 69 at 18 mo, and 86 at 25 mo.

tagenic agents. For example, despite changes in MF of less than twofold in Big Blue mice treated with *tris*(2,3-dibromopropyl)phosphate (de Boer *et al.* 1996b) or oxazepam (Shane *et al.* 1999), significant changes ($P = 0.02$ and $P < 0.015$, respectively) in MS were detected using the Monte Carlo test. *Tris*(2,3-dibromopropyl)-phosphate was found to induce a dose-dependent decrease in the frequency of G:C → A:T transitions (including the frequency of these mutations occurring at CpG sequences) and an increase in the frequency of deletions of G:C basepairs in the tumor target, but not nontarget, tissues. The *tris*(2,3-dibromopropyl)phosphate analyses and conclusions were subsequently confirmed using a log-linear statistical analysis (Brackley *et al.* 1999).

Stuart *et al.* (1996) used the Monte Carlo test to determine the influence of genetic background on *lacI* spontaneous MS recovered from the endogenous gene in *E. coli*, bacteriophage M13/*lacI*, λLIZ/*lacI* phage (*i.e.*, the Big Blue shuttle vector) propagated *in vitro* in *E. coli*, and *in vivo* and *ex vivo* λLIZ/*lacI* phage recovered from skin and liver of Big Blue mice. Despite the fact that all of the *lacI* genes included propagation through *E. coli* at some stage, by using the Monte Carlo test we were able to show that the MS segregated into four distinct groups: the *E. coli lacI* gene, M13*lacI*, λLIZ/*lacI* mutations arising *in vitro*/*ex vivo* during passage in *E. coli*, and λLIZ/*lacI* mutations arising *in vivo* in mouse skin and liver. Last, we note that Curry *et al.* (1999) used the Monte Carlo test to examine age-related changes in the human *HPRT* gene. They found that deletions >1 bp occurred twice as frequently in females as in males, but no other changes in MS with age were observed with the exception of A:T → C:G transversions, which increased in older individuals.

It is our experience (as well as that of other laboratories) that all mutagens, and even spontaneous mutations, exhibit unique MS (reviewed by Glickman *et al.* 1995); therefore, we are confident that age-related differences among MS from various tissues should be readily identified, should they exist. Last, we believe that even subtle differences among MS should be apparent upon careful examination of the spectra. This latter point is illustrated below during the discussion of increased frequency of TGG → TTT tandem transversion mutations in aging liver.

The data obtained in this study unambiguously demonstrated that spontaneous *in vivo* M*f* increased in aging mice in an adult somatic tissue that proliferates (liver) or is capable of proliferating when stimulated (bladder), but not in a nonproliferative tissue (brain; Figure 1; Table 1). M*f* increased at a relatively constant rate in liver of aging mice and at a significantly higher rate in bladder. Overall, at any age bladder M*f* were higher than those in liver, and liver M*f* were higher than those in brain. Compared with 1.5-month-old mice, liver M*f* increased 2-fold by 6 months of age and >3-fold by 25

TABLE 3

Spontaneous *lacI* mutations from bladder of Big Blue mice

	1.5 mo			6 mo			12 mo		
	No.	%	% at CpG	No.	%	% at CpG	No.	%	% at CpG
Transitions									
G:C → A:T	33	49.3	81.8	31	56.4	74.2	29	46.8	89.7
A:T → G:C	3	4.5		3	5.5		9	14.5	
Transversions									
G:C → T:A	18	26.9	61.1	9	16.4	44.4	11	17.7	54.5
G:C → C:G	1	1.5	0.0	0	0.0	0.0	2	3.2	50.0
A:T → T:A	5	7.5		4	7.3		0	0.0	
A:T → C:G	1	1.5		1	1.8		2	3.2	
Other mutations									
+1 frameshift	0	0.0		1	1.8		2	3.2	
−1 frameshift	3	4.5		4	7.3		7	11.3	
Deletions	3	4.5		1	1.8		0	0.0	
Insertions	0	0.0		1	1.8		0	0.0	
Complex changes	0	0.0		0	0.0		0	0.0	
Double mutants	0	0.0		0	0.0		0	0.0	
Total[a]	67	100		55	100		62	100	

[a] The total numbers of mutants after correction for clonality (see materials and methods). The noncorrected mutant totals were 71 at 1.5 mo, 64 at 6 mo, and 66 at 12 mo.

months of age. In bladder, M*f* in 12-month-old mice had increased almost 3-fold, relative to 1.5-month-old animals. A 1.6-fold increase in M*f* was observed in brain in maturing mice (1.5 months old compared with 6 months); however, after 6 months of age there was no further significant change in M*f* in adult brain. Collectively, these data suggest a correlation between cellular proliferation (nuclear DNA replication) and an increase in M*f*.

This study also describes the first detailed analysis of mutational spectra (specificity) as a function of age in selected tissues. As MS may provide insights into the origin of mutation, MS were determined for each tissue at each age (Tables 2–4). Interestingly, there were no significant differences in MS in mice of any age, indicating that the age-related increases in M*f* resulted from the accumulation of the same types of DNA damage by a pathway similar to that occurring earlier in life. This strongly hints that most, if not all, of the mutations that accumulate during aging share a common origin and are manifested through the process of cell proliferation. Specifically, these data suggest that there is no significant age-related accumulation of mutations that might be attributable to specific aging mechanisms, such as damage from free radicals, as this would result in changes in the relative proportions of the mutational classes that define the well-characterized spontaneous MS in younger animals (de Boer *et al.* 1997, 1998).

Of the three tissues examined, brain MS from mice of different ages were the most homogeneous, indicating that brain DNA was less affected mutationally by age than that of liver or bladder. Since M*f* in brain increased only ~1.6-fold (on average) after age 1.5 months (Table 1) with no change in MS, it seems probable that the mutations occurred primarily during DNA replication as brain tissue was proliferating, as it does early in life (Korr 1980).

The conclusion observed in this study that age-related effects on M*f* and MS in liver and brain accumulate during DNA replication is supported by the known proliferative activity of adult tissues. Liver is regarded as a slowly renewing (proliferating) tissue (Cameron 1970) in which DNA polyploidy levels steadily increase with age (Brodsky and Uryvaeva 1977; Enesco and Samborsky 1983), indicating that DNA replication is maintained in this tissue. Adult brain tissue consists primarily of nonproliferating neuronal cells, plus a much smaller population of glial cells (a fraction of which continue to proliferate in adults; Cameron 1970; Korr 1980; Korr *et al.* 1983). DNA content is also known to remain diploid in adult brain tissue (Winick *et al.* 1972). It should be noted that we do not suggest that the state of "being polyploid" itself increases M*f*; since M*f* are expressed as frequencies per 10^5 recovered transgenes, a simple doubling of the chromosome number by itself does not affect this ratio. However, the DNA replication that necessarily accompanies polyploidization provides additional opportunity for DNA lesions (or misincorporated nucleotides) to become established as mutations.

The elevated rate of increase in M*f* with age in bladder compared with liver was not predicted. Unstimulated urothelium of adult mice is practically mitotically quiescent, on the basis of the very low mitotic and labeling indices that are observed in this tissue (Clayson and Pringle 1966; Jost and Potten 1986; Jost 1989; Cohen and Ellwein 1991). Although mouse epithelial

TABLE 4

Spontaneous *lacI* mutations from brain of Big Blue mice

	1.5 mo			6 mo			12 mo			18 mo			25 mo		
	No.	%	% at CpG	No.	%	% at CpG	No.	%	% at CpG	No.	%	% at CpG	No.	%	% at CpG
Transitions															
G:C → A:T	19	42.2	73.7	28	43.8	64.3	24	39.3	91.7	21	50.0	76.2	18	51.4	83.3
A:T → G:C	3	6.7		3	4.7		3	4.9		2	4.8		3	8.6	
Transversions															
G:C → T:A	8	17.8	62.5	11	17.2	45.5	19	31.1	47.4	8	19.0	25.0	5	14.3	20.0
G:C → C:G	3	6.7	66.7	2	3.1	50.0	3	4.9	66.7	2	4.8	50.0	2	5.7	100.0
A:T → T:A	3	6.7		2	3.1		0	0.0		2	4.8		3	8.6	
A:T → C:G	0	0.0		3	4.7		2	3.3		0	0.0		1	2.9	
Other mutations															
+1 frameshift	4	8.9		5	7.8		3	4.9		2	4.8		0	0.0	
−1 frameshift	2	4.4		4	6.3		2	3.3		3	7.1		2	5.7	
Deletions	1	2.2		4	6.3		3	4.9		1	2.4		0	0.0	
Insertions	0	0.0		1	1.6		2	3.3		1	2.4		0	0.0	
Complex changes	1	2.2		1	1.6		0	0.0		0	0.0		0	0.0	
Double mutants	1	2.2		0	0.0		0	0.0		0	0.0		1	2.9	
Total[a]	45	100		64	100		61	100		42	100		35	100	

[a] The total numbers of mutants after correction for clonality (see materials and methods). The noncorrected mutant totals were 62 at 1.5 mo, 74 at 6 mo, 70 at 12 mo, 46 at 18 mo, and 39 at 25 mo.

bladder cells become polyploid, this process is essentially completed by ~6–8 wk of age (Walker 1958; Farsund 1975). Nevertheless, when the M*f* and MS from bladder are compared to those from liver and brain, it seems possible that DNA synthesis or cellular proliferation rates in the bladder may have been higher than expected, although the factors that may have contributed to such an increase in this study remain unexplained. However, it is noted that normal bladder function is significantly affected by a variety of stimuli, including diet, and bladder retains a capacity for rapid regeneration following mechanical trauma and chemical injury (Hicks 1975; Cohen 1995).

An alternative explanation for the enhanced rate of mutant accumulation in bladder follows from the observation that the frequency of "sectored" (*in vitro, ex vivo*) mutant plaques (Table 1) increased dramatically with age in bladder. These mutants, believed to result from expression in *E. coli* of unrepaired, damaged mouse DNA (Stuart *et al.* 1996), indicate that bladder DNA accumulated more damage compared to liver or brain. This damage would contribute to an elevation in M*f* when these lesions were expressed as mutations during DNA replication.

DNA replication in adult mouse liver is largely associated with polyploidization and is maintained at a relatively constant rate (Brodsky and Uryvaeva 1977; Enesco and Samborsky 1983). Since liver M*f* also increased at a similar rate, it seems likely that the increase in M*f* in adult liver reflected the accumulation of mutations during polyploidizing DNA replication. Liver M*f* increased 1.95-fold in mice aged 1.5–12 months (Table 1), while bladder M*f* increased 2.93-fold over the same period. However, since bladder tissue is known generally to proliferate more slowly than liver, and the sectored M*f* data indicated that bladder accumulated more DNA damage, it seems probable that DNA replicative activity was lower in bladder than in liver and that decreased DNA repair activity (or possibly, the efficiency of repair) in bladder resulted in elevated M*f* compared with liver.

In regard to spontaneous somatic mutations, it has been determined that about half of all spontaneous mutations observed in young mice arise during development, with approximately half of these mutations occurring *in utero* (Zhang *et al.* 1995). Those observations were confirmed in our study, since the M*f* increased rapidly, from essentially zero at conception (3 wk before birth), to between 2.9×10^{-5} and 5.6×10^{-5} depending upon the tissue by 1.5 months of age (Table 1). These data again demonstrate a relationship between cellular proliferation, the rates of which are maximum during development, and M*f*. Ames has also noted that "mitogenesis increases mutagenesis" (Ames *et al.* 1993; Shigenaga and Ames 1993).

As indicated earlier, there were no generally interpretable age- or tissue-related differences or trends among the various MS, following pairwise comparisons

of MS using the Adams-Skopek (Monte Carlo) algorithm. However, subtle differences in the frequencies of some mutations were nevertheless noted (Tables 2–4). Among the three tissues, the proportion of G:C → A:T transitions that occurred at CpG sequences was greatest in bladder (82%, average of all age groups), compared with liver (65%, average) and brain (78%, average). Double (tandem) mutations appeared most frequently in liver compared with bladder and brain. Interestingly, the frequency of TGG/CCA → TTT/AAA (*i.e.*, 5′-TGG-3′ → 5′-TTT-3′ or 5′-CCA-3′ → 5′-AAA-3′ on the opposite strand) tandem mutations increased in liver (at various sites in the *lacI* gene), from $\sim 0.054 \times 10^{-5}$ (on average) in liver ≤12 months old, to 0.43×10^{-5} (8-fold increase) at 18 months and 1.1×10^{-5} (20-fold increase) at 25 months (sequence data not shown). Except for a 5.9-fold increase in the M*f* for deletions in 25-month-old liver compared with the average M*f* from liver aged 1.5–18 months (1.3×10^{-5} and 0.22×10^{-5}, respectively), no increases in the frequency of deletions were otherwise observed among the three tissues. According to Tables 2 and 4, there appeared to be a slight age-related decrease in the proportion (as a percentage) of G:C → T:A transversions that occurred at CpG sequences in liver and brain. However, when M*f* were calculated, there was only a trivial increase in the frequency of these mutations in liver and a trivial decrease in brain. Last, the frequency of −1 frameshifts appeared to increase with age in bladder.

The factors that may have contributed to the subtle changes in MS in the oldest tissues remain speculative. The increased frequencies of GG/CC → TT/AA tandem mutations and deletion mutations were specific to liver of the oldest mice, 18 and 25 months old. [An increase in the frequency of GG/CC → TT/AA tandem transversions was also noted by Buettner *et al.* (1999) in the *lacI* transgene from aging mouse liver.] This tandem transversion is otherwise rarely observed in Big Blue; excluding the 14 mutants from this study and 3 mutants recovered from dietary-restricted mice aged 6–12 months (G. R. Stuart and B. W. Glickman, unpublished results), we have identified GG/CC → TT/AA mutations in only 30/17,016 (0.18%) sequenced spontaneous and induced Big Blue *lacI* mutants (de Boer 1995; J. G. de Boer and B. W. Glickman, unpublished results). Among our collection of sequenced *E. coli lacI* mutants, only 2/14,400 (0.01%) GG/CC → TT/AA tandem transversions have been identified (de Boer 1995; J. G. de Boer and B. W. Glickman, unpublished results).

The observation that 11/14 (79%) of the GG/CC → TT/AA tandem transversions involved TGG/CCA sequences (including 6/6 mutations recovered from 25-month-old mice) suggests that these otherwise infrequent TGG/CCA → TTT/AAA mutations might represent a mutational "signature" of an age-related change in mutational spectrum in older liver. It has been observed that GG/CC → TT/AA tandem transversions result when plasmids treated *in vitro* with acetaldehyde (Matsuda *et al.* 1998), acrolein (Kawanishi *et al.* 1999), or crotonaldehyde (Kawanishi *et al.* 1998) are permitted to replicate in human cells. Interestingly, these and other mutagens can arise endogenously from lipid peroxidation (Nath *et al.* 1996; Chung *et al.* 1999) and normal cellular metabolism (Ostrovsky 1986). Since acrolein-deoxyguanosine but not crotonaldehyde-deoxyguanosine adduct levels increase in liver of older rats (Chung *et al.* 1999), it is possible that the tandem GG/CC → TT/AA transversions observed in liver in our study were due to acrolein. It is also possible, however, that the tandem mutations and deletions observed in aged liver were attributable to a suspected slight increase in error-prone DNA polymerase activity or template-directed mutagenesis (Taguchi and Ohashi 1997; Hampsey *et al.* 1988), as suggested by the severalfold increase in the sectored M*f* in older liver (Table 1).

Ames has proposed that oxidative damage is a major contributor to aging (Adelman *et al.* 1988; Ames and Shigenaga 1992; Helbock *et al.* 1998). While Ames' predictions of a causal relationship between oxidative damage and aging (*e.g.*, a decline of mitochondrial function and other physiological changes) are probably valid, our data indicated a negligible effect of oxidative damage on nuclear DNA in liver, bladder, and brain of mice aged 1.5–25 months. During DNA replication, 8-oxo-2′-deoxyguanosine (8-oxoG) present in the template strand can mispair with adenosine, leading to G:C → T:A transversion mutations, while misincorporation of 8-oxoG as a substrate nucleotide can lead to A:T → C:G transversions (Cheng *et al.* 1992). Our data revealed no age-related increases in the occurrence of either G:C → T:A or A:T → C:G transversions in older mice compared with young mice (Tables 2–4). Indeed, the proportion of these mutations relative to other changes remained relatively constant in adult liver, bladder, and brain, suggesting that oxidative DNA damage is not a major contributor to M*f* or MS in nuclear DNA. These data also agree with results from a recent study that found no significant age effects for the levels of 10 different oxidatively induced base lesions in both mitochondrial and nuclear DNA from rat liver (Anson *et al.* 1999). It is possible, however, that 8-oxoG (and hence, 8-oxoG-derived mutations) accumulate to an appreciable level only in animals of advanced age (Hirano *et al.* 1996; Kaneko *et al.* 1997).

Other laboratories have demonstrated significant increases in M*f* with age in tissues in *lacI* and *lacZ* transgenic mice (*e.g.*, Lee *et al.* 1994; Ono *et al.* 1995; Dollé *et al.* 1997); however, none have sequenced sufficient randomly selected mutants to permit evaluation of changes in mutational specificity with age. Lee *et al.* (1994) reported a fourfold increase in *lacI* transgene MF (uncorrected for clonal expansions) in spleen of mice from birth to 25 months old. Their MS consisted

of 14% G:C → A:T transitions (with 33% of these occurring at CpG sequences), 5% G:C → T:A transversions, 18% G:C → C:G transversions, 27% double mutants, and 1.5% "size-change" mutants (determined electrophoretically) in mice aged 1–2 months (increasing to 12–19% size-change mutants in mice aged 3–24 months). These MS deviated significantly from spontaneous *lacI* MS from spleen, liver, lung, bone marrow, stomach, skin, and kidney of 3- to 12-wk-old Big Blue mice (de Boer *et al.* 1998), as well as spontaneous *lacI* MS in the endogenous *lacI* gene in *E. coli*, bacteriophage M13, and sectored (*in vitro*/*ex vivo*) Big Blue plaques (Stuart *et al.* 1996). Thus, the spleen MS reported by Lee *et al.* (1994) are enigmatic.

Studies using plasmid-based *lacZ* transgenic mice have also demonstrated significant age-related increases in MF in liver and spleen, but not brain (Dollé *et al.* 1997; Vijg *et al.* 1997). However, mutants were simply screened for large size changes on agarose gels, which indicated that ~50% of the mutants contained deletions and complex chromosomal changes (Gossen *et al.* 1995; Dollé *et al.* 1997; Vijg *et al.* 1997). Since only eight mutants were sequenced (Dollé *et al.* 1997), a detailed analysis of the effect of age on the mutational spectra was not possible. Although the elevated frequency of deletions/rearrangements observed by Vijg and colleagues might reflect the *in vivo* frequency of these mutations, our transgenic *lacI* data (this study; de Boer *et al.* 1997) as well as a meta-analysis of human *HPRT* mutations (Curry *et al.* 1999) indicate that the frequency of deletions from the *lacZ* plasmid transgenic assay could be overestimated. Similarly, it appears that large deletions, >2 kb in length, are rare in the human factor IX gene (Ketterling *et al.* 1994). Although the Big Blue assay is likely insensitive to the detection of large deletion events (as well as chromosomal rearrangements), deletions >2 kb have been recovered (Winegar *et al.* 1994; Mirsalis 1995; Buettner *et al.* 1996). Theoretically, *lacI* deletions up to ~7.5 kb should be detectable (Dycaico *et al.* 1994).

In conclusion, the data presented in this study demonstrated an age-related increase in the frequency of spontaneous mutations with no significant differences in mutational specificity in nuclear DNA from three somatic tissues from mice up to 25 months old. It seems probable that the age-related increases in the spontaneous mutation frequencies reflect endogenous DNA damage that was subsequently expressed as mutations following DNA replication. The increases in M*f* with age partly support the somatic mutation theory. However, the absence of significant changes in MS in older animals tends not to support aging theories that are based primarily on predicted increases of oxidative damage or the accumulation of genetic errors (error catastrophe) in nuclear DNA. Finally, the relatively small (severalfold) increases in M*f*, combined with the absence of significant changes in MS in older animals, indicate that spontaneous mutations are likely to have a modest influence on the aging process, at least until late middle age. In this regard, it should be noted that mice nullizygous for the mismatch repair gene *Pms2* show a 100-fold elevation in mutation frequencies in all tissues examined compared to both wild-type and heterozygous littermates, but develop normally and do not appear to age prematurely (Narayanan *et al.* 1997).

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