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Frequent spontaneous deletions at a shuttle vector locus in transgenic mice

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Transgenic mice carrying multiple copies of a recoverable lambda phage shuttle vector (λsupF) were constructed for the purpose of studying mutagenesis in a whole animal. Spontaneous mutations in rescued *supF* target genes from several different lines of transgenic mice were analyzed. One mouse line, 1139, was identified in which the frequency of spontaneous mutations was unusually high (3.15×10^{-4}), 20-fold higher than in other transgenic mice carrying a similar number of copies of the lambda transgene (~100). Over 75% of the spontaneous mutations from 1139 mice were found to be deletions, whereas mostly point mutations were recovered from the other mice. In 1139 no significant variation among adult tissues has been detected. However, embryonic tissue yielded a 3- to 4-fold lower frequency of mutations, most of which were point mutations rather than deletions. The frequency of mutations at another locus, the hypoxanthine phosphoribosyl transferase gene, was not elevated in fibroblast lines established in culture from the 1139 mice. Overall, these results suggest that the deletion mutagenesis affecting the transgene sequences in 1139 mice is a locus-specific effect occurring during growth and development. The increased mutagenesis could not be explained by the degree of methylation of the transgene sequences, since hypermethylation was seen in both 1139 mice and other mice with a low frequency of shuttle vector mutations. The integrated lambda vector DNA in 1139 mice was mapped to a single site on chromosome 7, but no mechanism for the mutagenesis was suggested by this localization. It is proposed that the lambda DNA may have either integrated into an unstable genomic site or created a newly unstable locus in the process of integration.

Introduction

Elucidation of the processes involved in spontaneous mutagenesis may add to our knowledge of carcinogenesis, developmental defects and aging. The analysis of spontaneous, as well as induced, mutations *in vivo* has been facilitated by the construction of transgenic mice carrying shuttle vector DNA in their genome (Gossen *et al.*, 1989, 1991, 1993; Summers *et al.*, 1989; Kohler *et al.* 1990, 1991; Malling and Burkhart, 1992; Gunther *et al.*, 1993; Hoorn *et al.*, 1993; Mirsalis *et al.*, 1993; Provost *et al.*, 1993; Tao *et al.*, 1993; Winegar *et al.*, 1994). The use of lambda phage as a shuttle vector for mutation detection was first developed in mouse fibroblasts (Glazer *et al.*, 1986) and was subsequently applied to transgenic mice (Gossen *et al.*, 1989; Summers *et al.*, 1989; Kohler *et al.*, 1990). The lambda DNA is introduced into the mouse cell or mouse oocyte DNA by standard transfection or microinjection techniques (Summers *et al.*, 1989), and rescue of the lambda vector DNA from within the mouse DNA is accomplished by incubation of the mouse DNA in lambda *in vitro* packaging extracts. These extracts can identify, cut out and package the lambda DNA from within the mouse DNA into viable phage particles for growth and analysis in bacteria (Glazer *et al.*, 1986). In this way, mutations in the reporter gene that occurred in the mice can be detected using *Escherichia coli* and phage genetic techniques (Glazer *et al.*, 1986). Transgenic mice have been used to study *in vivo* mutagenesis by a variety of genotoxic agents (Gossen *et al.*, 1989; Kohler *et al.*, 1991; Malling and Burkhart, 1992; Mirsalis *et al.*, 1993; Hoorn *et al.*, 1993; Provost *et al.*, 1993; Winegar *et al.*, 1994) and have been proposed as test systems for toxicologic analysis.

However, such shuttle vector constructs in transgenic mice are not only useful as biological dosimeters for genotoxic exposures but may also serve as valuable tools to probe factors that influence genomic integrity in the cells and tissues of a whole animal. The transgenic reporter constructs may be particularly useful in examining the importance of locus-specific effects within the mouse genomic DNA. Different sites can have significantly different methylation patterns and chromatin structures. These features may influence susceptibility to DNA damage and accessibility to repair activities. There are also gene-specific and strand-specific differences in repair that relate to transcriptional activity (Hanawalt, 1991). Mutagenesis of the integrated transgene may be affected by any of these factors.

Locus-specific differences within the mammalian genome are also manifest in the phenomenon of chromosomal fragile sites (Glover and Stein, 1988; Rassool *et al.*, 1991, 1992; Li *et al.*, 1993; Kuo *et al.*, 1994). These sites are prone to chromosome rearrangements (Glover and Stein, 1988; Rassool *et al.*, 1991, 1992; Kuo *et al.*, 1994) and may be preferred sites for integration of foreign sequences introduced into mammalian cells (Rassool *et al.*, 1991). While such heterogeneity within the genome may complicate efforts to use transgenic reporter constructs as models for typical human exposures to carcinogens, it highlights the potential utility of these constructs as probes of genome structure *in vivo*. The strategy of using transfected DNA as a probe has been exploited in studying the genome stability of mammalian cells in culture (Murnane and Young, 1989; Murnane, 1990a,b; Murnane *et al.*, 1990; Rassool *et al.*, 1991, 1992; Murnane and Yu, 1993). For example, Murnane and colleagues have used transfected DNA to identify and study loci prone to rearrangements (Murnane and Young, 1989; Murnane, 1990a,b; Murnane *et al.*, 1990; Murnane and Yu, 1993). Extension of this strategy to transgenic mice may reveal additional features of genomic plasticity, such as age-related and tissue-specific effects.

In their work with *lacZ*-containing transgenic mice, Gossen and colleagues demonstrated that the site of integration of

shuttle vector DNA can influence the spontaneous mutation frequency (Gossen *et al.*, 1991, 1993). One of the strains of transgenic mice that they constructed was found to have a spontaneous mutation frequency between 5.1×10^{-5} and 1.55×10^{-4}, depending on tissue type. This was significantly higher than the frequency seen in the same shuttle vector in other, independently derived *lacZ*-containing mouse lines (in the range of 1.0×10^{-5}). Almost all of the spontaneous *lacZ* mutations isolated from this strain of mice were determined to be point mutations, and the vector sequences were mapped genetically to the X-chromosome.

We report here the identification of a line of transgenic mice showing not only an unusually high frequency of spontaneous mutations in the shuttle vector transgene (3.15×10^{-4}) but also a predominance of deletions, as opposed to point mutations, in the rescued vectors. Analysis of the tissue specificity of the spontaneous mutagenesis revealed a high mutation frequency and a majority of deletions in all adult tissues analyzed from the lineage. However, fetal tissue yielded a different pattern, characterized by a 3- to 4-fold lower mutation frequency and a higher proportion of point mutations, suggesting that the transgene deletions observed in adult tissues accumulated during growth and development. The transgene sequences were mapped to a single site on chromosome 7. Based on a comparison of mutagenesis at two other loci in the mice, it is proposed that the high spontaneous mutation frequency is a locus-specific effect.

Materials and methods

Lambda vector construction and DNA preparation

The λsupF vector was previously constructed for use as a shuttle vector in mouse fibroblasts (Glazer *et al.*, 1986). Lambda DNA was prepared for injection into mouse oocytes by isolating phage particles via equilibrium centrifugation in a cesium chloride gradient (Sambrook *et al.*, 1989) and by using a Qiagen column to purify the λsupF DNA, as instructed by the manufacturer (Qiagen, Chatsworth, CA).

Transgenic mouse construction

Transgenic mice were constructed by microinjecting ~1000 copies of lambda vector DNA at a concentration of 25 μg/ml in a buffer of 5 mM Tris, pH 7.4, and 0.1 mM EDTA into the mouse oocytes, which were transferred into foster mothers for development into mice (Jaenisch, 1988). Manipulation of the mouse embryos was performed by the Transgenic Mouse Core Facility at Yale University.

Tail DNA dot blots

Screening of mice for the presence of the lambda vector DNA was accomplished using DNA isolated from tail biopsies. Clippings of 2–3 cm were treated with lysis buffer (50 mM Tris, pH 8.0; 100 mM EDTA; 100 mM NaCl; 0.5% SDS; 200 μg/ml proteinase K) followed by extraction with 1 vol of phenol, 1 vol of phenol:chloroform (1:1) and 1 vol of chloroform. The DNA was then precipitated with 2 vol of ethanol, air-dried and resuspended in 150 ml of TE buffer (10 mM Tris, pH 7.5; 1 mM EDTA). Five micrograms of DNA were applied to a nylon filter (Boehringer Mannheim, Indianapolis, IN) using a dot blot manifold (Schleicher and Schuell, Keene, NH). The filter was then air-dried and exposed to UV light to cross-link the DNA to the filter (Sambrook *et al.*, 1989). The presence of the lambda vector DNA in the mouse DNA was detected using ^{32}P-labeled λsupF DNA as a probe under standard hybridization conditions (Sambrook *et al.*, 1989). Copy number standards were prepared by dilution of a stock solution of λsupF DNA.

Lambda vector rescue and analysis

Packaging extracts for rescue of λ vector DNA were made as previously described (Glazer *et al.*, 1986), except that a new *E.coli* lysogen, NM759 [*E.coli* K12 *recA56* Δ(*mcrA*) e14° Δ(*mrr-hsd-mcr*) (λ*imm434 cIts b2 red3 Dam15 Sam7*)/λ], was used instead of BHB2690 for the preparation of the sonicate extract (Gunther *et al.*, 1993). This lysogen produces extracts that are deficient in methyl-directed restriction activity that would otherwise degrade DNA methylated in the mammalian pattern and reduce the yield of rescued phage (Gossen *et al.*, 1989; Kohler *et al.*, 1990; Gunther *et al.*, 1993).

DNA for vector rescue from the tissues of the transgenic mice was prepared using the same lysis buffer and purification method described above, except that prior mincing of the tissue samples with a sterile razor blade was performed. In the case of the mouse embryos, the entire embryos were minced, as the organs were too small to obtain distinct tissue samples. The mouse DNA was incubated in the lambda *in vitro* packaging extracts at a concentration of 0.05 μg/μl for 2 h at 37°C. The packaged phage were diluted in 10 mM Tris, pH 8.0, and 5 mM $MgCl_2$, adsorbed to PG901 [*E.coli* C1a *lacZ125* (*am*)] and plated in 0.6% top agar on LB plates in the presence of X-gal (1.6 mg/ml) and isopropyl-β-D-thiogalactopyranoside (IPTG) (1.3 mg/ml), as described (Glazer *et al.*, 1986). Phage with functional *supF* genes yield blue plaques, whereas mutants are colorless. To identify mutants in the *cI857* allele of the lambda repressor gene, the plates were incubated at 30°C (since this allele codes for a temperature-sensitive repressor). Repressor gene mutants are identified by a clear, as opposed to turbid, plaque phenotype (Wood *et al.*, 1984).

Mutation characterization

Following plaque purification, PCR amplification of the *supF* gene sequences was carried out using a GeneAmp kit (Perkin Elmer Cetus, Norwalk, CT). Primers were designed to generate fragments of either 250 or 700 bp encompassing the *supF* gene in the vector. A mutation was considered a deletion if no fragment was produced in four separate PCR reactions, two with each set of primers. To further evaluate the deletions, DNA from selected mutants was prepared from plate lysates using the Promega Magic lambda kit (Promega, Madison, WI), as directed. The phage DNA was digested with *Eco*RI and the products were analyzed by agarose gel electrophoresis, Southern blot hybridization and autoradiography using ^{32}P-labeled λsupF DNA as a probe. Rescued *supF* genes carrying point mutations as judged by PCR analysis were further examined by DNA sequence analysis of the PCR products, as described (Havre *et al.*, 1993).

Analysis of transgene methylation

The degree of methylation of the vector DNA in the different mouse lines was examined by comparing the extent of *Hpa*II and *Msp*I digestion of the integrated vector sequences. Samples of 10 μg of mouse genomic DNA were digested with 30 U of each enzyme for 18 h at 37°C. The products were analyzed by 1% agarose gel electrophoresis and Southern blot hybridization, using ^{32}P-labeled λsupF DNA as a probe.

Mouse cell lines

Preparation of primary cultures from male mice was performed as described (Sly and Grubb, 1979; Fischer *et al.*, 1980). Briefly, fresh tissue was coarsely chopped and placed in a dish under a slide coverslip. The samples were incubated in modified Eagle's medium supplemented with 10% fetal calf serum and 2× amino acids, 2× vitamins, 2× non-essential amino acids, penicillin and streptomycin (Gibco-BRL, Bethesda, MD) until outgrowth was detected, at which point the cells were scraped loose and transferred to fresh dishes. Expanded cultures were transfected with simian virus 40 (SV40) DNA (obtained from P.Gosh, Yale University) and, after several passages, pooled cells were analyzed for mutations in the HPRT gene using 6-thioguanine selection. Cells were plated at a density of 10^4 cells/cm^2 in the presence of 6-thioguanine at a concentration of 5 μg/ml. After 2 weeks, surviving colonies were visualized and enumerated by staining with crystal violet (72% methanol, 10% formaldehyde, 0.25% crystal violet). For analysis of *supF* mutagenesis in culture, clonally derived cell cultures were established by limiting dilution of SV40-transformed cultures in growth medium in 24-well plates. Single colonies were expanded under standard growth conditions, and DNA was prepared from at least 4×10^7 cells for phage rescue and analysis, as above.

Chromosome mapping of transgene locus

Chromosome analysis was performed on SV40-transformed fibroblasts isolated from mouse skin biopsies. Giemsa banding of chromosome spreads was performed as described (Shaper *et al.*, 1992). The locus of integration of the transgene vector sequences was determined by fluorescence *in situ* hybridization (FISH) analysis of previously G-banded chromosome preparations by the method of Lichter *et al.* (1988, 1990), using digoxigenin-labeled λsupF DNA as a probe and detected by anti-digoxigenin-conjugated rhodamine and 4′,6-diamino-2-phenylindole (DAPI) counterstain, as described.

Statistics

Mutation frequencies were compared pairwise by chi-square analysis with one degree of freedom, with the Yates correction factor.

Results

Transgenic mouse construction

A lambda phage vector, λsupF, was constructed to contain the *supF* amber suppressor tRNA gene of *E.coli* along with the *cI857* allele of the lambda repressor gene as mutation reporter genes. This shuttle vector has been used in mouse fibroblasts

in culture to study UV and X-ray mutagenesis (Glazer *et al.*, 1986; Yuan *et al.*, 1995). To construct transgenic mice, ~1000 copies of the λsupF vector DNA were microinjected into fertilized mouse oocytes, which were transferred to foster mothers for development into mice. Pups carrying lambda vector sequences were identified by dot blot analyses of DNA from tail biopsies. Positive mice were maintained and bred for further experiments. Figure 1 shows an examination of the presence of lambda vector sequences in one founder mouse, 1139, and in 11 pups in the F_1 generation derived from breeding 1139 with a non-transgenic mouse. By comparison with copy number standards prepared by dilution of lambda DNA, it was estimated that the 1139 founder contained ~100 copies of the vector DNA. As seen in this blot, the offspring

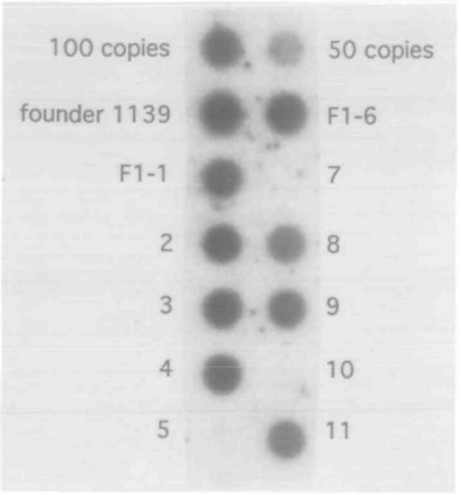

Fig. 1. DNA dot blot analysis of the presence of λsupF vector DNA in founder mouse 1139 and its F_1 progeny. Five microgram samples of DNA isolated from tail biopsies from the indicated mice were applied to nylon filters. Visualization of the presence of vector DNA within the mouse DNA was accomplished by hybridization with ^{32}P-labeled λsupF DNA, followed by autoradiography. As standards for quantitation, DNA from non-transgenic mice was mixed with purified λsupF DNA to approximate 50 and 100 copies of the vector DNA per mouse cell genome.

inherited either the full complement of transgene sequences or none, suggesting that all of the copies of the vector DNA were integrated at a single locus. Overall, 17/27 (63%) of the F_1 mice were positive for the transgene, consistent with Mendelian inheritance. Father-to-son transmission was observed, ruling out X-linkage.

By similar DNA dot blot analyses, two other founder mice, 1054 and 1230, were found to carry ~100 and 80 copies of the lambda shuttle vector DNA respectively (data not shown).

Chromosomal integration site

Further examination of the integration pattern of the transgene sequences was undertaken using the technique of FISH (Lichter *et al.*, 1988, 1990). A culture of fibroblasts was established from a skin biopsy derived from one of the 1139 F_1 mice. The cells were used for preparation of chromosome spreads. Using λsupF DNA as a fluorescently tagged hybridization probe, only a single locus was visualized, suggesting that all the transgene sequences are integrated at a single site. Sequential Giemsa banding and FISH analysis of the chromosomes revealed that the locus of integration is on chromosome 7 (Figure 2). A novel Giemsa staining band is produced by the vector sequences which is consistent with the presence of 100 copies of the 48 500 bp lambda vector (a total of 4.8 million new base pairs, constituting 0.1% of the transgenic mouse genome).

Spontaneous mutation frequency

In order to establish a baseline for studying induced mutagenesis and to begin to explore mechanisms of endogenous mutagenesis, we examined the spontaneous mutation frequency in the 1139, 1054 and 1230 mice. DNA was prepared from various tissues of adult mice that were grown under standard conditions. The mice were not specifically exposed to any known mutagen or other noxious agent. The mouse DNA was used as a substrate in lambda *in vitro* packaging extracts to rescue the λsupF vector DNA from within the mouse DNA for analysis. For this purpose, the extracts were prepared from a new *E.coli* lysogen with reduced methyl-dependent DNA restriction activity which would otherwise limit the yield of

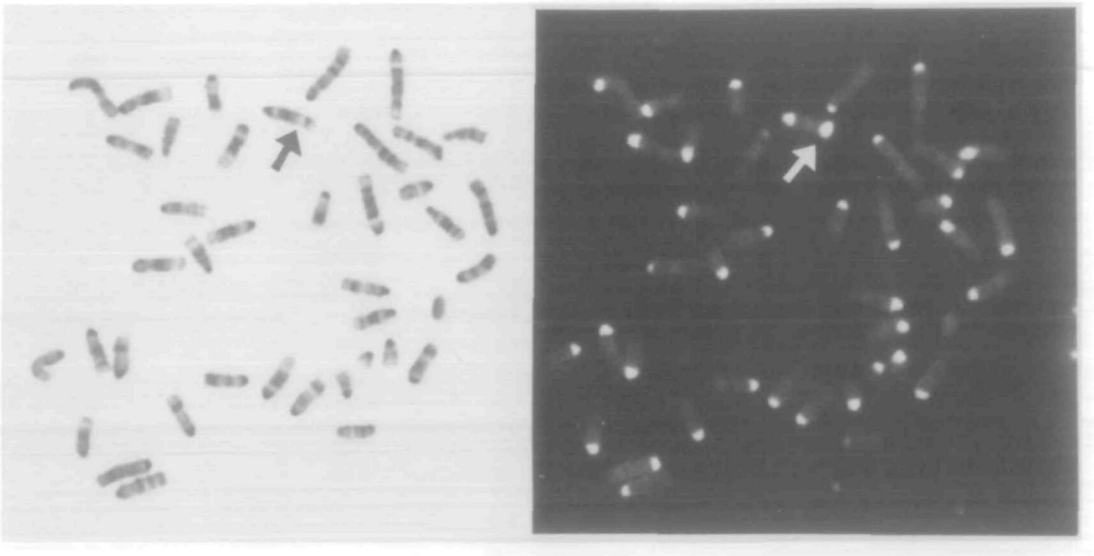

Fig. 2. Analysis of the chromosomal site of integration of the λsupF vector DNA in 1139 mice. Chromosome spreads were prepared from skin fibroblasts and used for sequential Giemsa banding (left panel) and FISH analysis (right panel). A novel hypochromatic band on chromosome 7 (black arrow) is visualized in the Giemsa-banded preparation. This correlates with the position of the FISH-localized λsupF DNA sequences (white arrow). The counterstain in the FISH analysis allows visualization of all chromosomes, in addition to the specific hybridization signal.

Table I. Frequency of mutations in λsupF shuttle vectors rescued from transgenic mice

Mouse line	Tissue	Mutation frequency ($\times 10^{-5}$)	Mutants per total plaques	% point mutations	% deletions
1139	skin	38.9	199/511 952		
	kidney	18.7	30/160 456		
	liver	29.3	47/199 006		
	spleen	35.3	82/232 583		
	bone marrow	25.4	25/98 460		
	brain	30.4	68/223 418		
	testes	30.9	112/362 984		
	total	31.5	563/1 788 859	11 (41/362)	89 (321/362)
1054	skin	1.3	10/761 144		
	kidney	1.7	4/241 375		
	liver	1.1	3/267 194		
	total	1.3	17/1 269 713	76 (13/17)	24 (4/17)
1230	skin	1.7	2/117 123	100 (12/12)	0 (0/12)
LN12 cells	fibroblast cell line	3.2	29 /892 700	100 (28/28)	0 (0/28)

phage rescued from DNA methylated in the mammalian pattern (Gunther *et al.*, 1993). Phage carrying mutations in the *supF* gene were identified by their inability to suppress the *lacZ* (amber) mutation in the host bacteria.

The mutation frequencies for the three mouse lines are listed in Table I. Also presented is the frequency of spontaneous mutations seen in a line of mouse fibroblasts (LN12) carrying ~100 copies of the same λsupF vector (Glazer *et al.*, 1986; Summers *et al.*, 1989; Yuan *et al.*, 1995). These cells were derived from mouse L cells and were previously constructed for studying mutagenesis in mammalian cells (Glazer *et al.*, 1986; Summers *et al.*, 1989; Yuan *et al.*, 1995). As seen from Table I, the frequency of spontaneous mutations in 1139 mice is 18- to 24-fold higher than in the other mouse lines ($P < 0.0001$) and 12-fold higher than in the LN12 cells ($P < 0.0001$). The elevated frequency of spontaneous mutations was seen in all 1139 adult tissues analyzed.

A high frequency of spontaneous mutations in the 1139 line was also seen to persist in all progeny of the founder that were tested over several generations. The frequency per 10^5 in the founder was 31.2; 34.2 in the F_1 generation; 39.2 in F_2; 24.1 in F_3; and 26.3 in F_4.

Analysis of mutations

The molecular nature of the mutations recovered from the mice was investigated. Initially, we attempted PCR amplification of the *supF* gene sequences in the mutant phage, to be followed by direct DNA sequencing. However, most of the phage mutants failed to yield a detectable fragment in the PCR reaction as analyzed by agarose gel electrophoresis, even after four attempts with two different sets of primers (data not shown). Such mutant phage were interpreted as having deletions of the *supF* gene sequences, and they were further analyzed by restriction enzyme analysis. DNA was prepared from the mutant phage, and the pattern of fragments generated by *Eco*RI digestion of the phage DNA was determined (Figure 3). To aid in the visualization of the restriction fragments, Southern transfer and hybridization with ^{32}P-labeled λsupF DNA were performed, followed by autoradiography. From the 48.5 kb phage DNA, the expected sizes of the fragments are 21.7, 19.9 and 6.9 kb. The internal 6.9 kb fragment contains the *supF* gene, and it represents the plasmid construct that was originally inserted into λgt2 to construct λsupF (Glazer *et al.*, 1986). It contains no essential phage genes. The 21.7

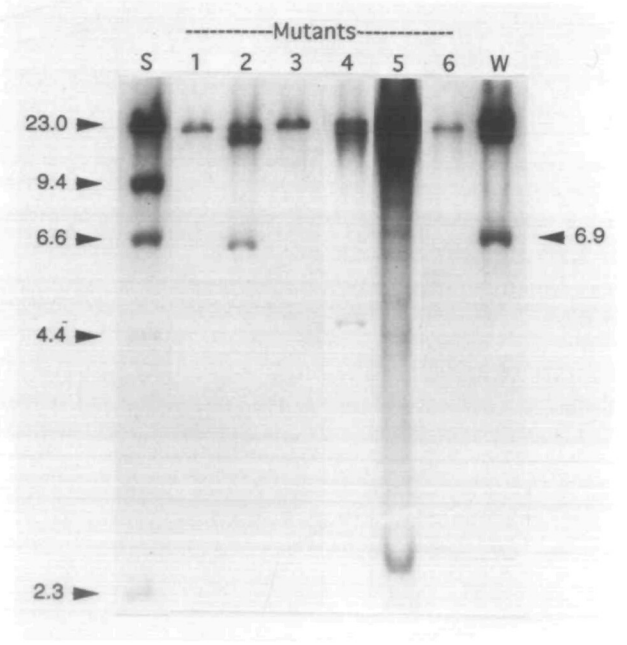

Fig. 3. Restriction enzyme digestion and Southern blot analysis of the spontaneous λsupF mutations recovered from 1139 mice. DNA was prepared from six mutant phage plus the wild type vector (W) and digested with *Eco*RI, followed by agarose gel electrophoresis, Southern transfer, hybridization to ^{32}P-labeled λsupF DNA and autoradiography. The expected pattern of bands (21.7, 19.9 and 6.9 kb) is seen in the wild type vector. The position of the *supF*-containing band in the wild type (6.9 kb) is indicated to the right, along with the size standards (S) on the left. Alterations in the pattern of *Eco*RI fragments in the mutant samples are indicative of deletions, as discussed in the text.

and 19.9 kb fragments represent the phage arms. An analysis of six spontaneous mutants rescued from 1139 mice is shown (lanes 1–6), in comparison with the wild type λsupF phage (W). In lanes 2, 4 and 5 the internal *Eco*RI fragment is visualized, but its size is seen to vary. This is consistent with the occurrence of deletions involving the *supF* gene and surrounding sequences within that fragment. The mutants in lanes 1, 3 and 6 have only a single large band, and so they represent deletions encompassing not only the *supF* gene and adjacent DNA but also both of the flanking *Eco*RI sites.

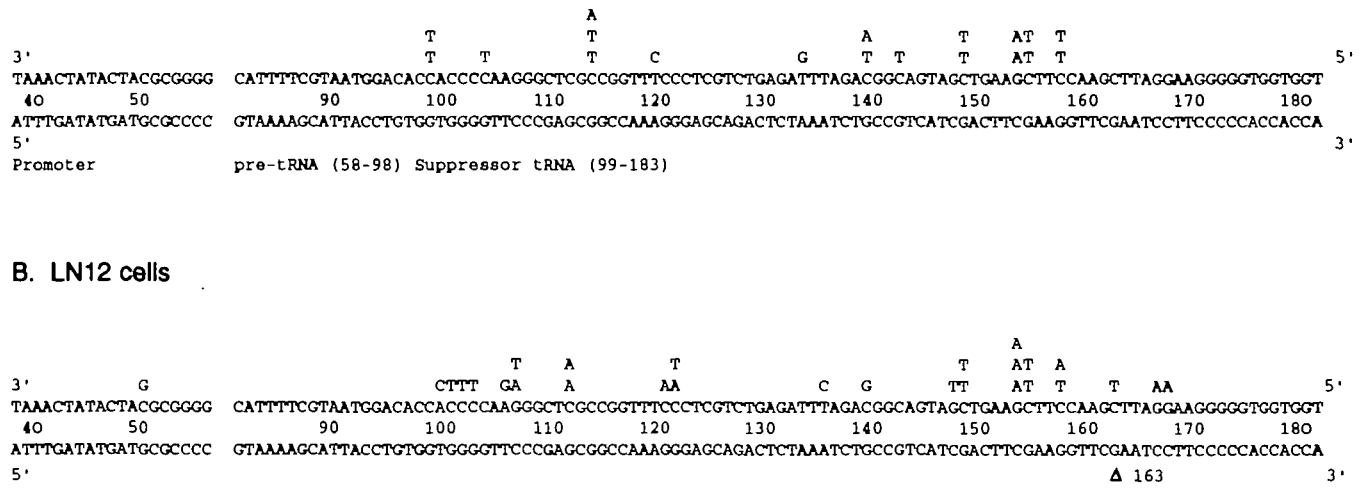

Fig. 4. Sequence analysis of spontaneous point mutations in shuttle vectors isolated from 1139 mice and from mouse LN12 cells.

Deletions >10 000 bp would not be visualized in this system, since such phage vectors would not survive.

Overall, 89% of the mutants rescued from 1139 were seen to carry deletions, whereas only 24% of the mutants rescued from the 1054 mice had deletions (Table I; $P < 0.0001$). All of the spontaneous mutations in phage rescued from the 1230 mice and the LN12 cells were point mutations. The observation of 24% deletions in the 1054 mice in the setting of an otherwise low overall spontaneous mutation frequency suggests that qualitative differences in the mutations can occur even among mice with low spontaneous mutation frequencies. In other reported transgenic mouse lines constructed with the *lacZ* and *lacI* reporter genes, most of the spontaneous mutations are point mutations (Gossen *et al.*, 1989, 1991, 1993; Kohler *et al.*, 1991). This includes the high spontaneous mutation frequency mouse ('35.5') identified by Gossen *et al.* (1991, 1993). Hence, the process of spontaneous mutagenesis in 1139 mice is unusual in that it generates a high proportion of deletions. The unusually high mutation frequency in 1139 mice is due almost entirely to the deletions. The absolute frequency of point mutations is $11\% \times 31.5 \times 10^{-5} = 3.5 \times 10^{-5}$, similar to the frequency of point mutations in the 1054 and 1230 mice. It might be argued that in the transgenic mice the multiple copies of the lambda DNA carried in tandem are predisposed to deletions via intrachromsomal recombination events. However, the deletions are not frequent in the other transgenic mice that we and others have produced, and so the presence of multiple copies of lambda DNA in the mouse genome does not necessarily lead to such deletions.

Analysis of point mutations

In order to determine if the unusual spontaneous mutagenesis in the 1139 mice might also affect the types of point mutations arising in the mice, we analyzed a sample of 19 point mutations isolated from 1139 mouse skin by DNA sequencing (Fig. 4). We compared these with a series of 29 spontaneous point mutations derived from the LN12 mouse fibroblasts. We found that all the point mutations from 1139 were single base pair substitutions, as were 28/29 from the LN12 cells. (There was one single base pair deletion found in the LN12 cell group.) In addition, the spectra of base substitutions in the 1139 and LN12 series were similar, with C:G to T:A transitions predominating. Hence, an unusual pattern was not seen in the 1139 point mutations.

Table II. Locus-specific comparison of the spontaneous mutation frequency in 1139 mice

Gene	Source	Locus	Mutation frequency ($\times 10^{-5}$)
supF	1139 mouse DNA	λ vector on chr. 7	31.5
cI	1139 mouse DNA	λ vector on chr. 7	45.5
supF	1054 mouse DNA	λ vector, locus not det.	1.3
cI	1054 mouse DNA	λ vector, locus not det.	2.8
HPRT	fibroblasts from 1139	X-chromosome	3.1
HPRT	fibroblasts from non-transgenic mouse	X-chromosome	2.0

Locus-specific effect

In seeking to determine the etiology of the spontaneous mutations in the 1139 mice, we asked whether the elevated mutation frequency was a generalized process occurring in the mice and mutating other genes (perhaps due to disruption of a gene involved in DNA repair by the insertion of the transgene sequences) or was an effect particular to the transgene locus. To address this question, we examined the frequency of spontaneous mutations in the *cI* lambda repressor gene (carried in the lambda vector DNA along with the *supF* gene) and in the hypoxanthine phosphoribosyl transferase (HPRT) gene (an endogenous mouse gene present on the X-chromosome). Mutations in the *cI* gene were identified by the morphology of the plaques formed by the rescued phage (Wood *et al.*, 1984). As shown in Table II, a high frequency of mutations, similar to that seen in the *supF* gene, was detected in the rescued *cI* genes.

To measure mutagenesis at the HPRT locus, fibroblast cultures were established from skin biopsies from male 1139 mice, as well as from non-transgenic mice as controls. Individual clones were not isolated; rather, pooled cells from each biopsy were seeded at $10^4/cm^2$ and were grown in the presence of 6-thioguanine, which is toxic to wild type cells containing

functional HPRT genes. Cells with HPRT gene mutations survive in the presence of 6-thioguanine and are detected by colony formation. The protocol yields a mutation frequency and not a mutation rate. It was designed to determine the proportion of mutations present in a sample of cells from the mouse tissues. We did not establish and expand clones from single cells because then we would be measuring mutagenesis that occurred during growth in culture rather than in the mice. The results indicate that the frequency of HPRT mutations in cells from the 1139 mice was not elevated, being similar to that seen in cells from non-transgenic mice (Table II).

The elevated mutation frequency in 1139 mice was therefore seen only in the reporter transgenes (*supF* and *cI*) carried in the lambda vector DNA on chromosome 7, not in the HPRT gene on the X-chromosome. These results suggest that the high spontaneous mutation frequency in 1139 mice is a locus-specific effect involving the transgene vector DNA and does not arise from an overall defect in DNA repair, replication or recombination that would affect other sites.

Embryonic versus adult tissue

Because a similar high spontaneous mutation frequency was seen in the 1139 founder transgenic mouse, as well as in all progeny tested, it was hypothesized that the mutations were somatic in origin and were not produced during meiosis. Otherwise, the founder would not show the effect, since the transgene sequences did not go through meiosis in the generation of the founder. Based on this hypothesis, we speculated that the mutations might arise either during the multiple rounds of replication that take place during mouse growth and development or during the cell replication required for tissue homeostasis in the adult animal. We asked whether there would be a detectable difference in the frequency of mutations present in embryonic versus adult tissue. DNA was prepared from 12-day-old embryos for analysis by shuttle vector rescue. Because of the small size of the embryos, specific tissues were not separated and multiple embryos were combined. An analysis of *supF* gene mutations in the vectors rescued from the embryonic tissue in comparison with the adult tissue is shown in Table III. The frequency of mutations in the embryos was 3- to 4-fold lower than in the adult tissue from 1139 mice and was more in line with the frequencies seen in other mouse lines. Note that this experiment measures mutation frequencies and not mutation rates, and so a 3-fold difference by itself may have minimal biological significance, although the data are highly statistically significant ($P < 0.0001$). However, most of the mutations from the embryos were point mutations and not deletions, in contrast to the 1139 adult tissue, indicating a qualitative difference between mutations found in embryonic and adult tissue.

We next asked whether the locus-specific deletion mutagenesis might take place when cells from the 1139 mice replicate during clonal expansion in culture. SV40-transformed fibroblasts from 1139 were cloned by limiting dilution. DNA was prepared from the cells after clonal growth to a total of ~4×10^7 cells. The *supF* mutation frequency in the phage rescued from these samples was found to be 3- to 4-fold lower than that seen in vectors rescued from the mouse tissue DNA (Table III). As with the embryonic tissue, most of the mutations were point mutations and not deletions. Hence, replication of adult tissue-derived cells *per se* does not yield the unusually high mutation frequency. Taken together, we interpret these results to suggest that the frequent deletion mutations observed in *supF* transgenes rescued from 1139 adult tissues are likely to have arisen during particular stages of growth and development.

Transgene DNA methylation

In considering mechanisms by which the deletions at the transgene locus might be generated, we examined the possibility that there might be a difference in the extent of cytosine methylation of the vector DNA in the 1139 mice as opposed to the other mice and mouse cell lines. For example, Engler *et al.* (1993) have reported that the methylation patterns of immunoglobulin gene constructs in transgenic mice can influence recombination events. To investigate cytosine methylation, mouse or mouse cell DNA samples were subject to digestion with *Hpa*II (which cuts at CCGG but is inhibited by

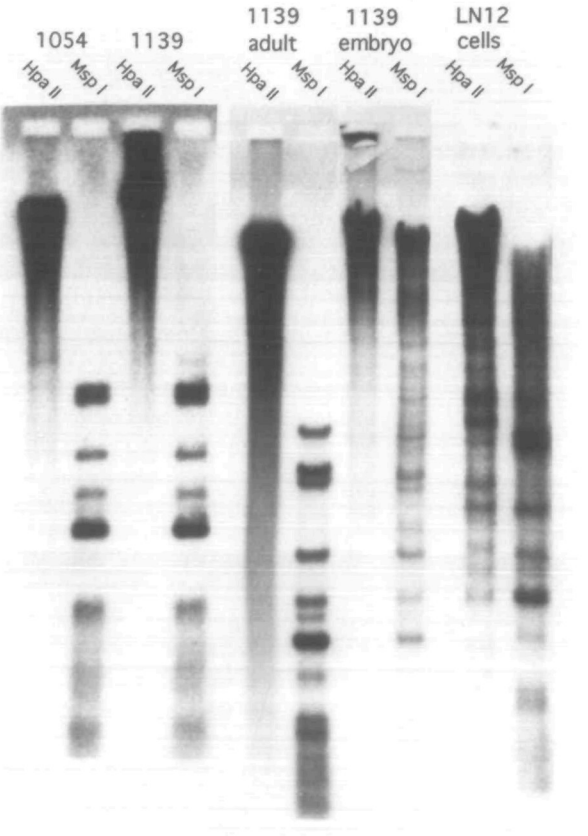

Fig. 5. Analysis of cytosine methylation of λsupF sequences within transgenic mouse DNA. Ten microgram samples of genomic DNA were prepared from adult 1054 and adult 1139 mice, from 12-day 1139 embryos and from LN12 cells (mouse fibroblasts transfected with the λsupF vector DNA). The DNA samples were digested with isoschizomers *Hpa*II and *Msp*I, as indicated, and the products were analyzed by agarose gel electrophoresis, Southern transfer, hybridization to ^{32}P-labeled λsupF DNA and autoradiography. Cytosine methylation at the internal C in CCGG sequences is indicated by inhibition of digestion with *Hpa*II. Hypermethylation is seen in all samples except the LN12 cell DNA.

Table III. Spontaneous mutation frequency in 1139 adult and embryonic tissue

Tissue	Mutation frequency ($\times 10^{-5}$)	Mutants per total plaques	% Point mutations	% Deletions
Adult	31.5	563/1 788 859	11 (41/362)	89 (321/362)
12 day embryos	9.8	38/385 965	88 (15/17)	12 (2/17)
Cloned adult fibroblasts	8.5	15/177 351	86 (12/14)	14 (2/14)

methylation of the second C) and with *Msp*I (which also cuts at CCGG but is insensitive to methylation at the second C). The digested samples were analyzed by agarose gel electrophoresis, Southern transfer and hybridization to ^{32}P-labeled λsupF DNA, followed by autoradiography (Fig. 5). Adult 1139 DNA (lanes 2 and 3) was compared with adult 1054 DNA (lane 1), embryonic 1139 DNA (lane 4) and LN12 cell DNA (lane 5). There was essentially complete *Msp*I digestion of all samples. *Hpa*II digestion of the transgene sequences was inhibited in 1139 adult, 1054 adult and 1139 embryonic DNA, indicating that the vector DNA was heavily methylated at the CCGG sites in all three samples. On the other hand, there was considerable digestion of the LN12 cell DNA by *Hpa*II, consistent with hypomethylation of the λsupF vector DNA in this cell line. Based on these results, it appears that the deletion mutagenesis is not correlated with cytosine methylation in these mice.

Discussion

Although the etiology of the high frequency of spontaneous mutations in the 1139 mouse line has not been established, several conclusions can be drawn from the data reported here. The occurrence of frequent mutations in the shuttle vector transgene is not related to the presence of multiple tandem copies of the shuttle vector in the mouse genome. Like 1139, the 1054 and 1230 mice carry multiple copies of the vector, but the transgene sequences in these other mice do not undergo such increased spontaneous mutagenesis.

Most of the mutations rescued from 1139 mice were deletions of various sizes. The requirements of the lambda shuttle vector are such that deletions >10 000 bp cannot be detected, and so the observed mutation frequency may be an underestimate. Also, we cannot distinguish between deletions occurring within a single copy of the vector DNA or those resulting from a recombination event between nearby copies of the vector DNA within the mouse chromosome.

The elevated mutation frequency was seen in all 1139 adult tissues analyzed, as well as in all generations of 1139 mice tested. A high mutation frequency was seen in both the *supF* and *cI* genes (carried in the lambda vector DNA on chromosome 7) but not in the HPRT gene on the X-chromosome. Hence, we conclude that the increased mutagenesis in the 1139 mice is a locus-specific effect involving the transgenic vector sequences. Embryonic tissue was found to have a 3-fold lower spontaneous mutation frequency than adult tissue and to contain mostly point mutations, not deletions, suggesting that most of the spontaneous deletions arise during some phase of growth and development. However, expansion of cloned fibroblasts from the 1139 mice yielded a low frequency of spontaneous mutations, most of which were point mutations. Therefore, cell replication is not by itself sufficient to induce the frequent deletions.

The question remains as to what is special about the transgene locus in 1139 mice that makes it prone to deletions. Theoretically, the activity of nearby genes might influence the stability of the transgene sequences. For example, Umar *et al.* (1991) observed increased mutagenesis of a *supF* reporter gene inserted next to immunoglobulin genes capable of rearrangement. In the 1139 mice the transgene sequences were mapped to chromosome 7. However, the mouse immunoglobulin genes are on chromosomes 6, 12 and 16 (Tonegawa, 1983), and so no obvious mechanism for the instability of the transgene sequences in 1139 is suggested by their localization to chromosome 7.

Another possible explanation is that the lambda vector DNA integrated into a fragile site in the mouse genome. Fragile sites have been observed in human and rodent cells and are associated with chromosomal instability, including chromosomal rearrangements, sister chromatid exchanges and intrachromosomal deletions (Glover and Stein, 1988; Rassool *et al.*, 1991, 1992; Kuo *et al.*, 1994). Also, the propensity of transfected sequences to integrate into fragile sites has been established in tissue culture experiments, and integration of foreign sequences into certain fragile sites can perturb these sites and lead to enhanced instability (Rassool *et al.*, 1991, 1992).

Apart from integrating into a pre-existing fragile site, the vector sequences may have created *de novo* an unstable site in the genome in the process of integration. For example, Murnane and colleagues (Murnane and Young, 1989; Murnane, 1990a,b; Murnane *et al.*, 1990; Murnane and Yu, 1993) have reported that integration of transfected plasmid DNA in mammalian cells can generate regions of persistent chromosomal instability.

If integration of novel sequences in transgenic mice can generate unstable loci, then there may be some degree of instability of transgene sequences in many of the transgenic mouse lines that have been constructed. At present, there is no evidence to suggest that the locus-dependent instability of transgene sequences in 1139 mice reported here is a phenomenon common to other types of mouse constructs. However, this instability would not be noticed in the typical transgenic mouse since most have not been designed to report mutations. Also, the mutation frequency observed here of 3.15×10^{-4}, while high for spontaneous mutagenesis, would not yield a detectable effect on the expression of the typical transgene. Still, out of eight shuttle vector-containing mouse lines reported (Gossen *et al.*, 1989, 1991, 1993; Kohler *et al.*, 1990, 1991; this work), two have shown elevated mutation frequencies [the 1139 line reported here and the 35.5 line of Gossen and colleagues (Gossen *et al.*, 1991, 1993)].

The exact nature of the vector DNA locus in 1139 mice remains speculative. The work reported here does not bear on the characteristics of the transgene locus other than the observation that the vector DNA is hypermethylated, as in other mouse lines. A further analysis will require cloning and comparison of the lambda vector integration sites in the several mouse lines. Nonetheless, the observation of locus-specific deletion mutagenesis in 1139 mice highlights the heterogeneity within the mammalian genome and suggests the utility of transgene vector constructs as *in vivo* genomic probes. The variability in spontaneous transgene mutagenesis seen in the mice reported here and elsewhere (Gossen *et al.*, 1989, 1991, 1993; Kohler *et al.*, 1990, 1991) also raises the possibility that induced mutagenesis in such mice may be similarly variable and locus-dependent. Further work is needed in this regard. In light of this possibility, however, caution should be exercised in interpreting mutagenesis experiments based on a single transgenic mouse line.

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