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Immunology

Mutation in a reporter gene depends on proximity to and transcription of immunoglobulin variable transgenes

(tRNA/shuttle vector/mutation assay/transgenic mice/κ light chain)

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ABSTRACT **Somatic mutation in immunoglobulin genes is localized to a 2-kilobase region of DNA surrounding and including rearranged variable (V), diversity, and joining (J) gene segments encoding heavy and light chains. To examine the structural basis for targeted mutation, we developed an assay to score mutation on plasmid substrates by using a reporter gene: a bacterial gene encoding an amber-suppressor tRNA molecule was placed 3′ of a rearranged κ VJ gene within the boundaries of mutation. The reporter gene is exquisitely suited for mutational analysis because it is only 200 base pairs (bp), which should not greatly disrupt structure of the immunoglobulin locus, and gene function depends on secondary structure, which means mutation can be scored in many different nucleotide positions. The plasmid was used to make transgenic mice, which were then immunized. The shuttle vector was retrieved by plasmid rescue into an indicator strain of *Escherichia coli* that contained an amber mutation in its β-galactosidase gene. Integrity of the tRNA molecule was monitored by colony color, which permitted many transformants to be screened visually. Mutations were not seen in DNA from a transfected B-cell line grown *in vitro* or in DNA from nonlymphoid tissue of transgenic mice, indicating that the reporter gene was stable during cell division and DNA manipulations. However, when the transgenic mice were immunized, DNA from splenic B cells contained point mutations in the reporter gene at a frequency of 10^{-3} per transformant. Sequence analysis of 17 mutated transgenes revealed that the mutations were 1- and 2-bp deletions in the tRNA gene, and one plasmid had an additional 2-bp deletion in the V gene. In contrast, previous studies have shown that mutations in endogenous VJ genes are predominantly nucleotide substitutions and have only 6% deletions. Two other plasmid constructs were analyzed in transgenic lines: no mutations were found when the tRNA gene was placed distal to the VJ gene, and no mutations were seen when the immunoglobulin promoter was deleted. Although we lack direct evidence that the deletions in the tRNA gene are caused by the same mechanism that acts on VJ genes, we have shown that mutations in this assay occur in a manner consistent with immunoglobulin-specific mutation in that they are found in splenic B cells and not in tail tissue, depend on position next to the VJ gene, and require transcription of the VJ gene.**

A hallmark of somatic mutation in murine immunoglobulin genes is the localization of mutation to rearranged variable (V), diversity (D), and joining (J) gene segments encoding heavy and light chains. Mutations are found at a frequency of ≈1% per nucleotide within a 2-kilobase (kb) region surrounding and including rearranged V(D)J genes but do not occur around the constant (C) gene (1, 2). The asymmetric distribution implies that there is a structural basis, such as cis DNA sequences, that targets the mutational process to the V(D)J gene. Recent analyses of mutations flanking rearranged V_H and V_κ genes indicated that the structural information for mutation is located in sequences around and within the V(D)J gene, rather than in sequences downstream of J gene segments (3, 4). The 5′ boundary was delimited by the promoter region, which suggests that transcription is required for mutation. The 3′ boundary was ≈1 kb from the V(D)J gene, and the frequency decreased with increased distance from the gene, implying that proximity to the V(D)J gene is necessary for nucleotides to mutate. The high frequency of mutations in the 3′-flanking region provides strong evidence that the mechanism is from point mutation rather than gene conversion because the 3′ intron is present in the genome as a single copy and has no homologous donor for conversion. The spectrum of 210 flanking mutations included 195 substitutions, 13 deletions of 1–14 nucleotides (nt), and 2 insertions of 2 and 4 nt. Most mutations were unique and occurred at different positions, which is consistent with a model for random point mutations.

To identify the molecular basis for localization of mutation to the V(D)J region, it is necessary to have both a simple, genetic assay to detect mutation and a system that can generate mutation in artificial substrates. A genetic assay that positively selects for mutation is essential for studies where the frequency of mutation is likely to be reduced—for example, when portions of the V(D)J gene and flanking sequences are deleted. Mutations must then be generated in the manipulated genes. Although there are no B-cell lines that sustain mutation at a high frequency *in vitro* (5), genes can be introduced into the germ line of mice, which are then immunized to initiate mutation. In fact, a striking frequency of mutation has been observed in transgenic mice by Storb and colleagues (6) in a κ light chain gene and by Durdik *et al.* (7) in a heavy chain gene.

We have developed an assay to score mutation by using a reporter gene placed next to a rearranged VJ gene encoding κ light chains within the boundaries of mutation described above. Mutation was assayed after immunization of transgenic mice. The ability to manipulate genes in a system where they undergo mutation allowed us to analyze several different constructs to determine why mutation is targeted to the VJ gene. The results show that mutation in the reporter gene (*i*) is tissue specific, (*ii*) depends on its position proximal to the VJ gene, and (*iii*) requires transcription of the VJ gene.

MATERIALS AND METHODS

Plasmid Constructs. A mutational substrate was derived from a productively rearranged κ gene with no mutations

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Abbreviations: nt, nucleotide(s); V, variable; D, diversity; J, joining; C, constant.

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from hybridoma HPCG9 (2). The genomic DNA clone contained 1 kb of 5′-flanking sequence including promoter and leader regions, a V_κ167 gene segment from the V_κ24 family that was productively rearranged to J_κ5, 2.5 kb of intervening sequence, the C_κ gene, and 1.2 kb of downstream sequence containing a poly(A) addition site. A natural *Bam*HI site between the leader and variable gene was removed and recreated 700 base pairs (bp) upstream. A suppressor tRNA gene (*S*), a 200-bp *Eco*RI fragment encoding an amber-suppressor tyrosine tRNA (*supF*) from *Escherichia coli* (8), provided by M. Seidman (Otsuka Pharmaceutical, Rockville, MD), was inserted at a unique *Xba* I site located 283 bp 3′ of J_κ5. The construct also included 1.7 kb of DNA, containing a gene conferring resistance to ampicillin, and a ColE1 origin of replication derived from pBR322 for growth in bacteria. The plasmid was 7.3 kb in size and contained a single *Bam*HI site. Four plasmids, displayed in Fig. 1, were made from this basic mutational substrate as follows. Plasmid pVSC-GB contained an additional 13.4-kb *Bam*HI fragment with the *gpt* gene encoding xanthine-guanine phosphoribosyl transferase and bovine papilloma virus (BPV) (9) for selection and integration into hybridoma cells. Later experiments showed that bovine papilloma virus sequences were not necessary for integration. Plasmid pVxSC was engineered by oligonucleotide-directed mutagenesis (10) to contain a stop codon (x) at amino acid 49 in the V_κ-coding region. A deletion of thymine at position I of the codon encoding amino acid 23 also occurred during the construction. Plasmid pVxCS had the tRNA gene inserted 5.7 kb 3′ of the nonfunctional VJ gene. Plasmid pP^-VSC had 700 bp deleted 5′ of a functional VJ gene starting at nt 326 upstream of amino acid 1.

Hybridoma Transfection and Transgenic Mice. For hybridoma transfection, pVSC-GB was transfected into the SP2/0 B-cell line (11) by protoplast fusion using polyethylene glycol (12) and selected for expression of the *gpt* gene product in the presence of mycophenolic acid. A cell line called SP2.C5 was selected for further experiments. For transgenic mice, pVxSC, pVxCS, and pP^-VSC plasmids were linearized with *Bam*HI and injected into pronuclei of $B6AF_1 \times$ C57BL/6 embryos. Transgenic mice were maintained as backcrosses to C57BL/6 animals; the mice used in this study were from the fourth generation. The number of plasmids integrated into genomic DNA from SP2.C5 cells and the tails of transgenic mice was estimated by scanning radiographs from Southern blots hybridized with a C_κ probe with a densitometer and comparing intensity of the integrated and endogenous bands. Southern blots were also hybridized with a probe containing the bacterial origin of replication and ampicillin-resistance gene; the hybridizing bands comigrated with those detected with C_κ, indicating that smaller plasmid fragments had not integrated into chromosomal DNA.

RNA Analysis. Nuclei were isolated from the following hybridomas: SP2.C5, HPCG13 (2), and a hybridoma from each transgenic line. Nuclear run-off transcription was performed by incubating the nuclei with [α-^{32}P]UTP and unlabeled ATP, GTP, and CTP and allowing transcription to occur (13). Approximately 10×10^6 cpm of labeled RNA was hybridized to nitrocellulose containing 2 μg of immobilized plasmids with the V_κ167 gene segment and a murine β-actin gene. The autoradiograph was exposed for 3 weeks, and hybridization was quantified by densitometry. For analysis by PCR, first-strand cDNA was made from nuclear run-off RNA by using a forward C_κ oligonucleotide, and the product was amplified with a reverse V_κ167 primer by using a GeneAmp kit (Perkin–Elmer/Cetus). The primers were 20 nt long, were annealed at 59° for 2.5 min, and were elongated at 72° for 1 min. Amplification continued for 36 cycles. Amplified products were separated by electrophoresis on a 5% acrylamide gel, stained with ethidium bromide, and visualized with UV light.

Mutation Assay. Three- to six-month-old transgenic mice were injected i.p. with a mixture of antigens containing 33 μg each of alum-precipitated phosphorylcholine–bovine serum albumin, dinitrophenyl–hemocyanin, and fluorescein–hemocyanin and 10^9 *Bordetella pertussis*. Seven days after the primary or secondary injection, spleen cells were prepared, and T cells were removed with anti-Thy1.2 and complement. Genomic DNA was made from the remaining B cells, and plasmids were rescued as follows. Ten micrograms of DNA was digested with *Bam*HI and 2 μg of RNase, treated with phenol and ether, precipitated, resuspended, and self-ligated in 400 μl. Ligated DNA was precipitated and resuspended in 50 μl of H_2O. Five microliters containing 1 μg of DNA was transformed by electroporation into *E. coli* strain MLB100, obtained from M. Seidman, using a Bio-Rad Gene Pulser apparatus (Bio-Rad Laboratories, Richmond, CA) and a technique that yielded $>10^9$ transformants per μg of supercoiled plasmid DNA (14). Bacteria were plated on agar plates in an overlay of 3 ml of 0.7% agar containing 150 μg of ampicillin, 600 μg of isopropyl β-D-thiogalactoside and 1 mg of 5-bromo-4-chloro-3-indolyl β-D-galactoside. White colonies containing potential mutations were replica-plated, grown on nitrocellulose, and hybridized to a V_κ167 probe. In a typical DNA transformation, $\approx$1% of the colonies were white and did not hybridize to the V probe; these colonies had large deletions of the V and tRNA genes and were not analyzed further. Colonies that hybridized were grown, plasmid DNA was prepared, and the size was determined by restriction analysis on agarose gels. Restriction fragments from plasmids with the correct size were then subcloned into M13 phage and sequenced using the dideoxynucleotide chain-termination method with appropriate primers.

RESULTS

Copy Number and Transcriptional Status of Integrated Plasmids. To determine whether the tRNA marker gene is stable during cell division *in vitro*, the gene was transfected into SP2/0 hybridoma cells and tested for mutation. A cell line called SP2.C5 was chosen that contained $\approx$150 copies of

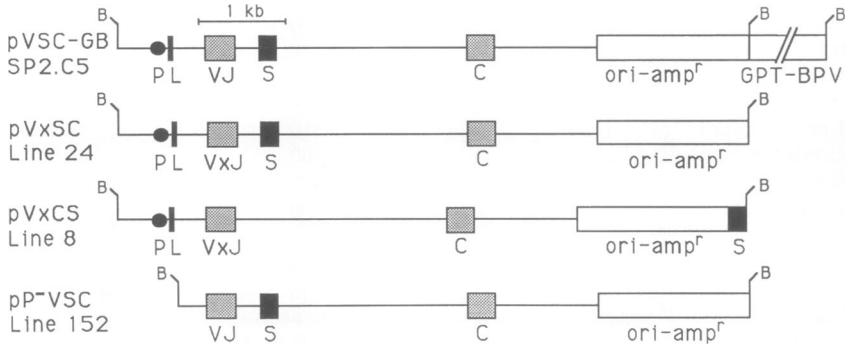

FIG. 1. Plasmid constructs for mutational analysis. The plasmids contain a promoter (P), leader sequence (L), V_κ167 gene segment rearranged to J_κ5 gene segment, suppressor tRNA gene (S), C_κ gene (C), bacterial origin of replication (ori), and ampicillin-resistance gene (amp^r). The second and third constructs have a stop codon (x) in the V gene segment. pVSC-GB has an additional 13.4-kb fragment containing the *gpt* gene and bovine papilloma virus sequences. B, *Bam*HI. Name of cell or transgenic lines containing constructs is noted below plasmid.

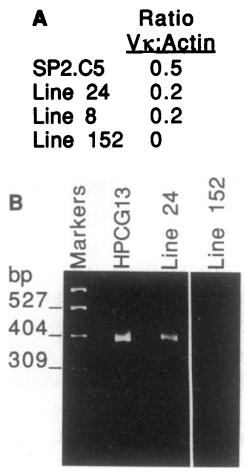

FIG. 2. Transcription of transfected plasmids and transgenes. (*A*) Ratio of nuclear run-off transcription of V_κ167 vs. β-actin, as measured by densitometric scans. (*B*) Gel analysis of size of nuclear run-off transcripts amplified by PCR with V_κ167 and C_κ primers. Gel was stained with ethidium bromide.

the pVSC-GB plasmid (Fig. 1) that were tandemly integrated into chromosomal DNA. Transcription of the transfected immunoglobulin genes was assessed by nuclear run-off assays, which measures the relative rate of transcription rather than accumulation of transcripts. Hybridization to V_κ167 DNA was assumed to be due solely to transcription of the transfected genes because there was no background transcription of V_κ167 in SP2/0 cells. The hybridization signal for V_κ167 was compared with the signal for an endogenous gene, β-actin, and is shown as a relative ratio in Fig. 2*A*. The transfected genes were transcribed in SP2.C5, although the transcription rate per gene copy appears considerably reduced relative to β-actin.

To generate mutation on substrates containing the marker gene, plasmids were integrated into the genome of embryos that were used to make transgenic mice. Three lines were produced from three plasmids, summarized in Fig. 1. (*i*) Line 24 contained the pVxSC plasmid, which had the tRNA gene placed proximal to the VJ gene. Seven copies of the plasmid were integrated and cosegregated through four generations, suggesting that they inserted into the same chromosome. Southern blot analysis showed that five copies were integrated in a tandem cluster, and two copies were separately integrated. Within the cluster, three internal copies were present as the original 7.3-kb size, and two flanking copies were on 9.4- and 10.1-kb *Bam*HI fragments, indicating that the flanking copies lost the original *Bam*HI site at integration and incorporated endogenous host DNA at the 5′ and 3′ ends. The two separately integrated copies contained the entire plasmid plus additional flanking host DNA and were present on 11.4- and 23-kb *Bam*HI fragments. Transcription was measured by both hybridization and amplification of run-off transcripts prepared from the nuclei of a hybridoma cell line. Hybridoma cells were used because there was no endogenous transcription of V_κ167 in the SP2/0 fusion partner. Fig. 2*A* shows that the V_κ167 gene was transcribed in line 24. To determine whether the transcript containing a tRNA gene in the intron is accurately spliced, the transcript was amplified by PCR with V_κ167 and C_κ primers that should yield an expected fragment of 380 bp. Fig. 2*B* demonstrates that the transgenic transcript is identical in size to an endogenous transcript from a rearranged V_κ167 gene in the HPCG13 hybridoma, indicating that the tRNA gene did not interfere with splicing. (*ii*) Line 8 contained the pVxCS plasmid, which had the tRNA gene placed beyond the boundaries of somatic mutation. Five copies were tandemly integrated into chromosomal DNA, and the V_κ167 gene was transcribed (Fig. 2*A*). (*iii*) Line 152 contained pP^-VSC plasmid, which had a functional V_κ167 gene and a deletion of 700 bp of 5′ DNA containing the leader and promoter regions. Approximately 15 copies were tandemly integrated. As expected, there was no transcription of the V_κ167 gene as measured by nuclear run-off (Fig. 2*A*) or PCR (Fig. 2*B*).

Assay for Mutation in Rescued Plasmids. High-efficiency rescue of the integrated plasmids from chromosomal DNA was achieved by electroporation into bacteria. Approximately 300 transformants per μg of splenic DNA were obtained. Restriction analysis of 30 plasmids selected randomly from line 24 indicated that most of the seven integrated copies were recovered: 66% of the plasmids were the 7.3-kb size that is present in three copies, 27% were the 9.4-kb size, 3% were the 10.1-kb size, 3% were the 11.4-kb size, and none were the 23-kb size. Lower recovery of the larger plasmids may be from decreased efficiency of transformation of large DNA or acquisition of restriction or methylation sites in host DNA that interfere with bacterial transformation (15).

Rescued plasmids were scored for mutation by using a procedure described by Seidman *et al.* (16). The reporter gene is a bacterial suppressor tRNA gene, present on a 200-bp fragment that contains a bacterial promoter for expression in bacteria. Suppressor function is measured in an indicator strain of *E. coli*, MLB100, which contains an amber mutation in its β-galactosidase gene. If the tRNA remains functional, the stop codon in the bacterial β-galactosidase gene is suppressed, and blue colonies are produced in the presence of isopropyl β-D-thiogalactoside and 5-bromo-4-chloro-3-indolyl β-D-galactoside. If mutation occurs in the tRNA gene to disrupt secondary structure, bacterial colonies will be white.

Frequency of Point Mutation in a Cell Line and Transgenic Mice. To determine the spontaneous frequency of point mutations in the transfected tRNA–immunoglobulin construct, DNA was analyzed from cells and tissues not expected to mutate. DNA from the SP2.C5 cell line was examined to assess the effect of *in vitro* division and transcription on frequency of mutation. Table 1 shows that 114,000 blue colonies were recovered, and no white colonies of the appropriate size were detected, indicating that the transfected plasmid had a very low frequency of spontaneous mutation of $<9 \times 10^{-6}$ per transformant. DNA was also

Table 1. Frequency of mutation in a reporter gene from a cell line and transgenic mice

Source of DNA	Blue colonies	White colonies*	White colonies, %
SP2.C5 cell line	114,000	0	<0.0009
Tail, line 24 (14 mice)†	17,886	0	<0.006
Tail, line 8 (9 mice)	15,124	0	<0.007
Tail, line 152 (3 mice)	12,052	0	<0.008
Splenic B cells, line 24			
1° mouse 1	1,750	8	0.5
1° mouse 2	1,720	0	<0.06
2° mouse 3	7,731	3	0.04
2° mouse 4	2,544	6	0.2
Splenic B cells, line 8			
1° mouse	2,850	0	<0.04
1° mouse	3,850	0	<0.03
2° mouse	1,700	0	<0.06
2° mouse	8,358	0	<0.01
Splenic B cells, line 152			
1° mouse	2,214	0	<0.05
1° mouse	13,310	0	<0.008
2° mouse	8,100	0	<0.01
2° mouse	15,422	0	<0.006
2° mouse	6,690	0	<0.01

1°, DNA made 7 days after primary immunization; 2°, DNA made 7 days after secondary immunization.
*White colonies that hybridized with V_κ167 and were the same size as control plasmids by restriction analysis.
†Group includes DNA from the tails of mice 1–4.

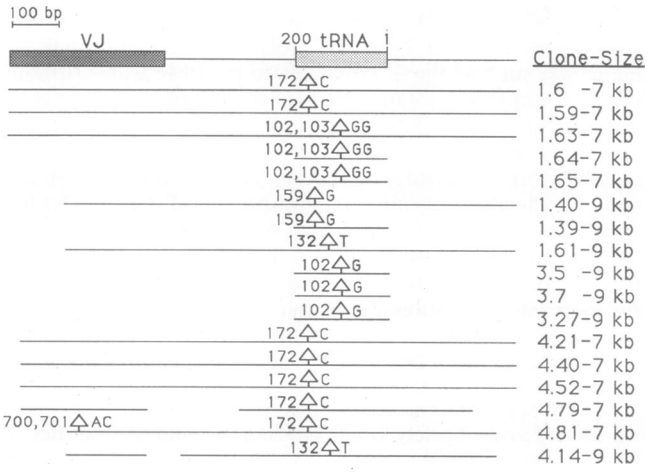

FIG. 3. Location and sequence of mutations in rescued plasmids from line 24 B cells. Top line represents the immunoglobulin germline sequence and the tRNA sequence. First digit of clone number corresponds to mouse number in Table 1, and size is shown as either 7 (7.3 kb) or 9 (9.4 kb). Horizontal lines represent distance sequenced. Position of mutations in the tRNA gene corresponds to numbering in ref. 8; position of the VJ gene mutation in clone 4.81 is numbered according to ref. 2. △, Deletion.

analyzed from tails of the three lines of transgenic mice, taken both at 4 weeks of age and when spleens were removed. Approximately 15,000 blue colonies were rescued from each line, and no white colonies were found. Thus, frequency of mutation was $<6–8 \times 10^{-5}$, indicating low mutation frequency in the tRNA gene from nonlymphoid tissue.

DNA from splenic B cells from the three transgenic lines was analyzed after primary and secondary immunizations. In line 24, one of two mice from a primary immunization had mutation at a frequency of 0.5%, and both mice from a secondary immunization had mutation at frequencies of 0.04% and 0.2%. Thus, from 13,745 recovered transgenes, mutation in the reporter gene was detected at an average frequency of 0.12%. The sequences of plasmids from 17 white colonies are shown in Fig. 3; all mutations were due to 1- and 2-bp deletions in the tRNA gene. The mutant plasmids were each derived from independent transformations, and many from the same mouse had identical mutations. One clone, 4.81, had a 2-bp deletion in the VJ gene in addition to the tRNA mutation. In contrast to the mutation detected in line 24, no white colonies were detected in a comparable number of transformants recovered from primary- and secondary-immunized B cells from line 8, where the tRNA gene was placed distal to the VJ gene (0 white/16,758 blues = <0.006%); nor were white colonies detected in line 152, where the immunoglobulin promoter was deleted (0 white/45,736 blues = <0.002%).

DISCUSSION

Development of an Assay to Score Mutation on Plasmid Substrates Containing Immunoglobulin Genes. As an initial step toward defining the structural requirements that target mutation to rearranged V genes, we designed a genetic assay that measures mutation in artificial substrates. The assay features a reporter gene to score mutation so that mutants can be easily identified, and the assay can be used for experiments where the coding region is mutagenized because it does not depend on function of immunoglobulin protein. The reporter gene is a bacterial tRNA molecule, well suited for mutational analysis because it has a small size, which should not greatly disrupt structure of the immunoglobulin locus, and the molecule has a high degree of secondary structure necessary for function, which means mutation can be scored in many different nucleotide positions. The suppressor tRNA gene has been used to study mutagenesis in mammalian cells (17), and point mutations have been detected in 74 of the 85 bases encoding the mature tRNA molecule (18). The integrity of the marker gene is monitored by colony color in bacteria, permitting visual screening of many transformants. Mutational substrates were constructed that contained the tRNA and immunoglobulin genes on plasmid shuttle vectors, which could be rescued from genomic DNA by digestion with a restriction enzyme and efficiently recovered by electroporation into an indicator strain of bacteria. Rescued plasmids were then sequenced to locate the mutations.

To determine the spontaneous level of mutation in the reporter gene during cell division, the pVSC-GB plasmid (Fig. 1), with the tRNA gene placed proximal to the VJ gene, was integrated into chromosomal DNA of the SP2/0 B-cell line. Cells were grown *in vitro* for several months, and the DNA was analyzed. No mutations were detected in the marker gene, analogous to the lack of mutation in endogenous V(D)J genes during tissue culture (5). Therefore, the reporter gene showed remarkable stability during cell division and manipulation of the DNA during restriction enzyme digestion, ligation, and transformation.

Mutation Occurred in a Tissue- and Site-Specific Manner. To generate mutation on the substrates, plasmids were introduced into embryonic DNA as transgenes and mutation was induced by immunizing the mice. Three different constructs (Fig. 1) were analyzed in separate transgenic lines: (*i*) pVxSC in line 24, to determine whether mutation could occur in a marker gene placed next to the VJ gene; (*ii*) pVxCS in line 8, to assess the effect of distance from the VJ gene on frequency of mutation; and (*iii*) $pP^{-}VSC$ in line 152, to see whether transcription of the VJ gene is necessary for mutation. Because the first two plasmids were constructed to contain a stop codon in the V gene segment and the third construct was not transcribed, no protein was expressed from the transgenes. There are several advantages to studying mutation in a silent gene: the immune repertoire of transgenic mice would be normal because V-to-J joining should occur on the endogenous alleles, and any antigen could be used to stimulate B cells through their endogenous receptors. Furthermore, mutation has been shown to occur at about the same frequency in nonproductively rearranged genes as in productively rearranged genes (19).

Mutation in the transgenes was detected in immunized splenic B cells from line 24 at a frequency of ≈0.1% per transformant. Seventeen bacterial transformants from three of four immunized mice had mutations in the tRNA gene; lack of increased mutation after secondary immunization may be from the small sample size of two mice in each category. Mutation was detected in both the 7.3- and 9.4-kb copies, which were recovered more frequently than the other three copies that integrated as larger *Bam*HI fragments. In contrast, no mutation was found in 17,886 transformants from tail DNA of line 24 mice, including the tails from mice that had mutation in their splenic B cells, which showed that the mutations were not present in the germ line. Thus, somatic mutation occurred in B cells at a frequency >20-fold over a nonlymphoid tissue, indicating that the mechanism functioned in a tissue-specific manner analogous to immunoglobulin-specific mutation.

Mutation also occurred in a site-specific manner because the reporter gene did not mutate in B cells from line 8 mice when the tRNA gene was placed 5.7 kb from the 3′ side of the VJ gene or 1 kb from the 5′ side if the transgenes integrated head-to-tail. We have previously defined the boundaries of mutation in endogenous V(D)J genes as ≈300 bp on the 5′ side and 1 kb on the 3′ side (3), which implies that the

mutational machinery operates for a limited distance and then stops.

Transcription Is Required for Mutation. A correlation between transcription and mutation has been implied indirectly from several findings. (*i*) Mutation does not occur at a high frequency in unrearranged V gene segments (20, 21), suggesting that the segment becomes more susceptible to mutation after rearrangement brings it near the enhancer, which increases transcription rate. (*ii*) The 5′ boundary is near the promoter, indicating that transcriptional machinery may be involved in delimiting a boundary or in generating mutations (3). To directly test whether transcription is necessary for mutation to occur, we compared the frequency of mutation in the reporter gene in line 24, which had an immunoglobulin promoter, and in line 152, which deleted the promoter. Line 24 transcribed the V and C transgenes as assessed by nuclear run-off transcripts from a hybridoma and PCR amplification. However, the transgenes were probably inefficiently transcribed because they lacked an enhancer located 3′ of the C_κ gene. The 3′ enhancer has been reported to increase transcription by 7-fold compared with an enhancer located in the V–C intron (22), which was present in our constructs. Different chromosomal sites of integration of the transgenes may inhibit transcription as well (23). Although mutation was detected in line 24, it occurred at a lower frequency than in endogenous VJ genes, suggesting that the decreased mutation frequency may be linked to the reduced transcription rate of the transgenes. Recent evidence of high frequency of mutation in κ transgenes containing the 3′ enhancer (24), but not in those without it (25), supports the correlation between transcription rate and mutation frequency.

Lack of mutation in the tRNA gene in line 152, which lacked the immunoglobulin promoter and had no detectable transcription of the transgenes, shows that transcription is required for mutation. However, although transcription is necessary, it does not appear sufficient to induce mutation because no mutation was detected in the SP2.C5 cell line, which transcribed the VJ and C genes. Thus, additional factors, such as T-cell interleukins, may be necessary to induce the mutational process.

Pattern and Frequency of Mutation in the Reporter Gene Indicate That Additional Elements Are Necessary. Three aspects of the pattern and frequency of mutation in the reporter gene were different from mutation in endogenous V genes. (*i*) All transgene point mutations were 1- and 2-bp deletions—in contrast to endogenous somatic mutations in flanking regions, which are predominantly nucleotide substitutions. It is unlikely that the deletions were introduced during the cloning procedure because no mutations were found in 221,556 plasmids ($<10^{-6}$) rescued from DNA of tissue culture cells, tails (including line 24), and splenic B cells of lines 8 and 152. (*ii*) Five positions repeatedly mutated among independently derived colonies, whereas endogenous mutations in DNA-flanking V(D)J genes showed no evidence of hot spots. (*iii*) Mutation frequency in the sequenced transgenes was 0.1% per nucleotide, compared with 0.8% in similar regions from endogenous V(D)J genes. Only one bacterial colony had a mutation in the VJ gene in addition to the tRNA mutation, in contrast to the multiple mutations seen in endogenous VJ genes.

The pattern of deletions and hot spots may be caused by the specialized sequence and secondary structure of the tRNA gene, which may favor certain types of mutation. The low frequency of mutation suggests that the mutational machinery is acting suboptimally on the transgene; for example, deletions may be due to an inefficient repair process, where nucleotides are removed by an exonuclease but the gap is not completely filled in by DNA polymerase. Additional cis elements, such as the 3′ enhancer to increase transcription, may be required to obtain the full spectrum of mutations seen in endogenous VJ genes. Other factors could also affect efficiency of the mutator process—i.e., the tRNA gene itself could disrupt structure of the rearranged immunoglobulin gene, or the chromosomal integration site of the transfected substrates may not permit a high mutation rate. Therefore, strategies for targeting genes into their natural locus through homologous recombination (26) may be valuable in the future for increasing mutational activity.

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