Somatic Hypermutation of an Immunoglobulin μ Heavy Chain Transgene

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Summary

We have analyzed somatic hypermutation of an immunoglobulin (Ig) heavy chain transgene. Hybridomas expressing the transgene were produced from immunized transgenic mice and transgene copies were sequenced to assay for mutation. In two IgM-producing hybridomas, as well as in several IgG-producing hybridomas, mutations were found in the VDJ region of the transgene. In the IgM-producing hybridomas, both mutated and unmutated transgene copies were present and expressed as mRNA. Several mutated transgene copies were present in a single cell and these showed different patterns of mutation. Two IgG-producing hybridomas isolated from a single animal also showed a hierarchical pattern of mutation indicating that transgene mutations can accumulate during B cell proliferation, similar to the mutational process for endogenous antibody genes. Among hybridomas that expressed both IgG and IgM molecules derived from the transgene, the isotype-switched γ transgene copy exhibited a higher level of mutation than the μ transgene copies. Our results indicate that the 15-kb ARSμ transgene contains all the sequence information required to target the Ig-specific hypermutational machinery, and raise the possibility that sequences associated with the endogenous CH locus might enhance somatic mutation.

Somatic hypermutation contributes to the diversification of the antibody H and L chain V(D)J gene segments and is important for affinity maturation of antibody responses (1–6). Despite the extensive evidence of Ig gene hypermutation, little is known about the sequences that target this process to the V(D)J regions of H and L chain genes. Mutations are localized within an ~2-kb region surrounding the V(D)J exon (7–11) of either productively or nonproductively rearranged genes (12–14). Mutations are rare in germline, V, J_κ, and J_H segments (15, 16). In addition, incomplete DJ-joined H chain alleles show levels of mutation much lower than found in comparable V(D)J-joined alleles (13, 17). These findings suggest that sequences associated with the V gene segment and the J gene segment might both be required for efficient hypermutation of a recombined Ig gene.

Rearranged H chain and κ L chain transgenes introduced into the germline of mice have also been shown to be substrates for somatic hypermutation (18–21). This indicates that transgenic mouse models might be used to localize the *cis*-acting Ig gene sequences required for targeting the hypermutational mechanism. We have previously reported that a μ H chain transgene can undergo isotype switching by an interchromosomal DNA recombination mechanism and that the transgene VDJ region was frequently somatically mutated in the switched transgene copy (21). This suggested that the transgene VDJ region might contain the sequence elements required to target the hypermutational mechanism. However, because the transgene VDJ region was juxtaposed to the endogenous CH locus in these switched transgene copies, it was not clear whether the sequences required to target hypermutation were provided by the transgene, the endogenous CH region, or both.

To determine whether the ARSμ transgene contains all the sequences required for targeting hypermutation, we have now analyzed the VDJ sequences of μ transgene copies in hybridomas that have not undergone isotype switching. We find that mutated μ transgene copies are present in IgM-producing hybridomas derived from immunized transgenic mice. Within individual hybridomas both mutated and unmutated transgene copies are present, different mutated transgene copies display different somatic mutations, and both mutated and unmutated transgene copies are transcribed. The transgene mutations that we detect in IgG- and IgM-producing hybridomas are point mutations localized largely to the VDJ region, and our results indicate that mutations can accumulate successively during cell proliferation. These features mimic normal Ig gene hypermutation and suggest that the same mechanisms are involved in hypermutation of both endoge-

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nous Ig genes and Ig transgenes. In hybridomas from immunized transgenic mice that express both IgM and IgG derived from the transgene, the isotype-switched γ transgene copies display significantly higher levels of mutation than the μ transgene copies. Based on our results, we conclude that the sequence elements required for targeting the hypermutational mechanism appear to be present on the 15-kb ARSμ transgene DNA segment. We also discuss models to account for the different levels of mutation in μ and γ transgene copies. One possibility that we consider is that sequences located near the Cγ region might enhance somatic hypermutation.

Materials and Methods

Transgenic Mice. Transgenic mice have been described previously (21). The 15-kb transgene is composed of a recombined VDJ gene isolated from the A/J-derived hybridoma R16.7 and BALB/c-derived Cμ. The transgene (ARSμ) encodes an Ig H chain which, when associated with an appropriate L chain, makes CRI_A positive anti-Ars antibody. All mice used in this study were derived from the ARS5 transgenic line, which contains ~30–50 copies of the transgene per cell (21). The transgene copies in ARS5 mice are located on mouse chromosome 5 and are arranged predominantly in a tandem head to tail array (21, 22).

Hybridomas and Antibodies. IgM hybridomas that were analyzed by RNase protection were obtained by fusing transgenic spleen cells with the Sp2/0-Ag14 myeloma line. (ARS5 × BALB/cJ)F_1 transgenic mice were immunized twice with 100 μg of ARS-KLH in CFA intraperitoneally and, 7 d before fusion, boosted intraperitoneally with 100 μg of ARS-KLH in saline. The hybridomas 1G10, 1E9, and 2B4 have been described previously (21). All IgG, IgM double-producing hybridomas (except 1G10) were also obtained from this fusion. The mAbs RS3.1 (23) and MB86 (24) were used to assay for μ^a and μ^b allotypes, respectively. Microtiter wells were coated with RS3.1 (5 μg/ml) or MB86 (10 μg/ml), exposed to samples, and developed using biotin-labeled RS3.1 or MB86 (200 ng/ml) and ^{125}I-labeled streptavidin. Test samples were compared with standards using TEPC 183 (μ^a) and H4.2.5 (μ^b) mAb, as well as BALB/c (μ^a) and C57BL/6 (μ^b) sera. Assays for anti-ARS reactivity, CRI_A idiotypes, and AD8 reactivity were described previously (21).

RNase Protection Assay. The in vitro synthesis of ^{32}P-UTP–labeled RNA probe, hybridization, and RNase digestion were performed according to the specifications of Promega Biotec (Madison, WI). The ^{32}P-labeled RNA probe was generated by transcribing the antisense strand of pGARP with SP6 RNA polymerase. pGARP contains the transgene VDJ in the pGemini (Promega Biotec) vector (see Fig. 2 *a*). Transcripts from this vector initiated from the T7 RNA polymerase promoter (the sense strand) gave no signal in protection assays with any of the RNAs used in this study. After phenol/chloroform extraction and ethanol precipitation, the RNA probe was resuspended in hybridization buffer (80% formamide, 40 mM Pipes, pH 6.7, 0.4 M NaCl, 1 mM EDTA). Hybridoma mRNA was added to the probe, hybridized at 42°C overnight, and the samples were treated with RNase A (40 μg/ml) and RNase T1 (2 μg/ml) for 30 min at 15°C. Digested hybrids were analyzed on a 6% denaturing acrylamide gel and exposed to X-ray film at −70°C.

cDNA and Genomic DNA Library Construction. To construct a cDNA library from hybridoma 2C7, 100 μg of 2C7 mRNA was used to synthesize oligo-dT–primed double-stranded cDNA using the Librarian™ cDNA library construction system (Invitrogen, San Diego, CA). cDNA fragments between 1.5 and 3.5 kb were purified from an agarose gel and ligated into bacteriophage λgt10. Approximately 6 × 10^4 recombinant phage were screened with ^{32}P-labeled V133, a probe containing 133 bp corresponding to amino acids 15–59 of V_H 36–65 (25). Purified phage clones from three separate plugs were analyzed.

To produce an EMBL3 genomic DNA library, 2C7 genomic DNA was digested with BgIII. DNA fragments of 12–19 kb were purified on an agarose gel and ligated into BamHI-digested EMBL3 arms. For the λgt10 2C7 genomic DNA library, 4.4–6.6 kb EcoRI fragments were ligated to λgt10 arms. Phage libraries were screened with ^{32}P-labeled J_H3-4 and V133 probes separately. Plaques that hybridized to both probes were purified and analyzed.

PCR, Cloning, and Sequencing. The transgene VDJ region was PCR amplified from either hybridoma genomic DNA or cDNA, using the primer pairs as shown in Fig. 1. Hybridoma cDNA was synthesized using the J_H2, μ, or γ primers, as required. PCR protocols of either 30 cycles of 1 min denaturation at 94°C, 3 min annealing at 57°C, and 2 min extension at 72°C, or 30 cycles of 1 min denaturation at 94°C, 2 min annealing at 55°C, and 3 min extension at 72°C were used. Genomic transgene clones in EMBL3 or λgt10 bacteriophage vectors were PCR amplified directly from the phage plug using primer sets specific for either the transgene VDJ region, the enhancer region, or the CH1 region (Fig. 1). PCR products were digested with the appropriate restriction enzymes, cloned in pUC18, and sequenced by the dideoxy method (26) using pUC sequencing primers. For the EMBL3 genomic clone, two PCR clones were sequenced and found to be identical. Transgene VDJ regions of λgt.2C7 clones were PCR amplified, and the PCR products were purified on an agarose gel and sequenced using the *f-mol*™ DNA sequencing system (Promega Biotec). For hybridomas expressing γ transgene copies, two PCR clones derived from γ cDNA were sequenced. In each case the two PCR clones were identical.

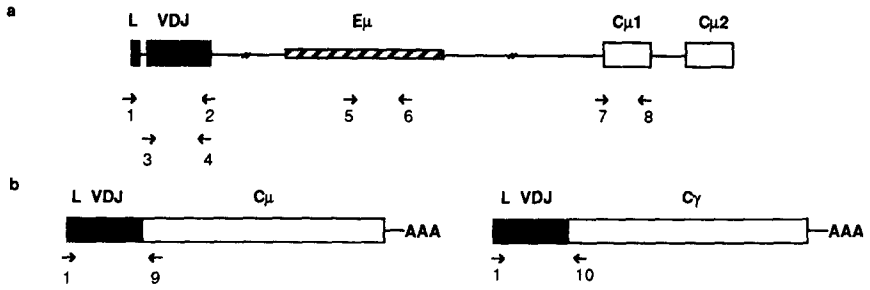
Denaturation Gradient Gel Electrophoresis (DGGE)[1]. The transgene VDJ regions of λgt.2C7 clones were PCR amplified for DGGE analysis. PCR conditions were as above. The two nested sets of primers are shown in Fig. 1. A 40 base GC-clamp was included in the internal 5′ primer of the nested PCR primer set to increase the sensitivity of the DGGE assay (27, 28). The PCR products were fractionated on a 4% polyacrylamide gel containing a 15% to 40% denaturant gradient. 100% denaturant was composed of 40% (vol/vol) formamide and 7 M urea. Electrophoresis was performed at 150 V for 3.5 h in a 60°C water bath. The gel was stained with ethidium bromide and the DNA was visualized with UV light.

Preparation of Metaphase Spreads and In Situ Hybridization. Cells from hybridomas 2C7.F5, 4C5.F11, and 5A2.A11 were harvested by standard methods and spread on microscopy slides. The ARSμ plasmid (21) was nick translated with Bio-dUTP (Sigma Chemical Co., St. Louis, MO) for in situ hybridization. Washing, blocking, detection, and amplification were described previously (22).

Results

Detection of Mutations in Transgene-encoded IgM by RNase Protection. To screen rapidly for mutations in IgM hybridomas from transgenic mice, we used an RNase protection assay which had the potential to detect mutations in one expressed copy of the transgene amidst a possible background

[1] *Abbreviation used in this paper:* DGGE, denaturation gradient gel electrophoresis.



c

1. F: 5'-GTGGAATTCAAACCATGGGATGGAGCTTC-3'
   EcoR1
2. R: 5'-CCAGAATTCACCTGAGGAGACTGTGAGAG-3'
   EcoR1
3. F: 5'-*CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCC*GCTTCTGGATATACATTC-3'
4. R: 5'-GTGCCTTGGCCCCAGTAG-3
5. F: 5'-GCGAATTCAAAGGAACACAGAAGTATG-3'
   EcoR1
6. R: 5'-GCAGGATCCATTTTAGAAGTCGATAAAC-3'
   BamH1
7. F: 5'-GGAATTCAAGGAAATAGCAGGGTGTAG-3'
   EcoR1
8. R: 5'-GCAGGATCCTCTGTTTTTGCCTCCGTAG-3'
   BamH1
9. R: 5'-CGAATTCGCTCTCGCAGGAGAC-3'
   EcoR1
10. R: 5'-GGAATTCCGGGGCCAGTGGATAGAC-3'
    EcoR1

Figure 1. PCR primers. (*a*) Primer set (*1,2*) was used for PCR amplification of the transgene VDJ from DNA templates. Primer sets (*5,6*) and (*7,8*) were used to amplify the μ enhancer and Cμ1 from the genomic phage clones containing transgene VDJ. Primer sets (*1,2*) and (*3,4*) were used to prepare samples for a DGGE by two rounds of sequential amplification of the transgene VDJ from recombinant λgt10 phages. (*b*) Primer sets (*1,9*) and (*1,10*) were used for reverse transcription and PCR amplification of the transgene VDJ from μ and γ RNA. (*c*) Nucleotide sequence of primers used for PCR. (*F* and *R*) forward and reverse primers, respectively. Restriction enzyme sites included in the primers are underlined. The GC clamp attached at the 5' end of the primer 3 is marked in italics.

of ~30–50 unmutated copies. The rationale for this assay was based on the known ability of bovine pancreatic RNase A to cut at single basepair mismatches in RNA:RNA hybrids (29–32). We tested this assay using RNAs from transgene-expressing IgG hybridomas in which the expressed VDJ sequence had previously been determined. Hybridomas 1E9, 1G10, and 2B4 have 1, 7, and 17 mutations, respectively, in the VDJ region of the expressed γ transgene copy (21, 22). Mutations are successfully detected by this assay in 1G10 and 2B4 (Fig. 2 *b*). Bands corresponding to protection of the probe over the entire VDJ region (378 bp) are not observed. Instead, smaller protection bands are found. The single C to G substitution present in 1E9, however, was not revealed on this assay by RNase protection (Fig. 2 *b*), probably because G:G mismatches are not cleaved by RNase A (32). Thus, although the protection assay cannot detect all point muta-

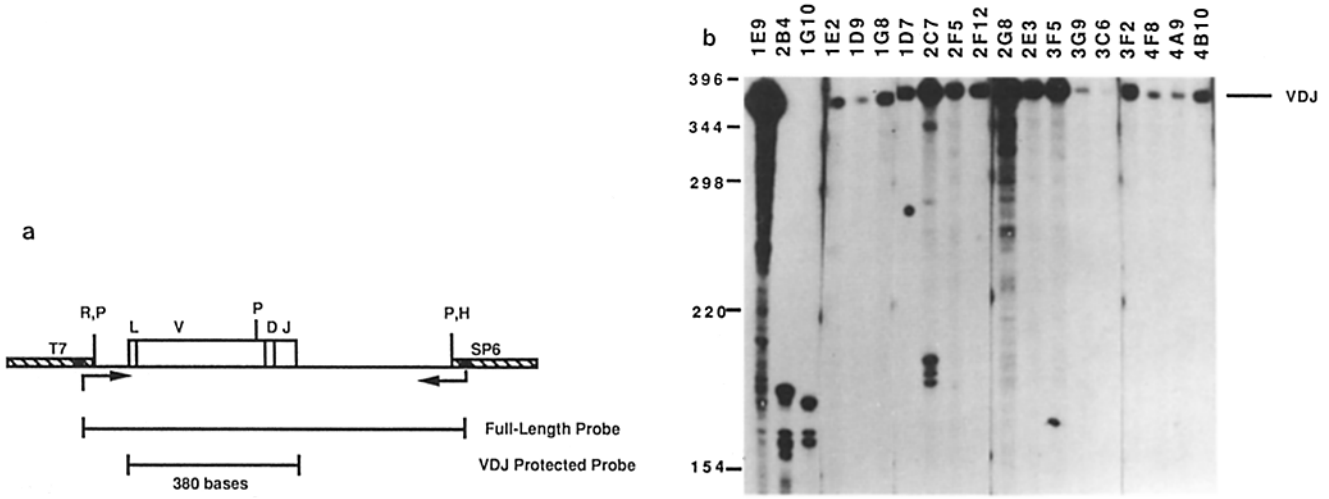


Figure 2. (*a*) Map of the transgene VDJ probe used for RNase protection experiments. Neighboring PstI fragments containing transgene VDJ sequences were cloned into pGem-3. This coding region is indicated by a white box; (*black lines*) intron sequences. (*Crosshatched*) Plasmid sequences. (*Arrows*) Direction of in vitro transcription from the bacterial promoters SP6 and T7 (*black boxes*). ^{32}P-labeled antisense transcripts generated from the HindIII-linearized plasmid using T7 polymerase did not protect mRNA from transgene-expressing cells (data not shown). (*b*) RNase protection analysis of ARS-reactive, transgene-expressing hybridomas. Hybridomas 1E9, 2B4, and 1G10 are somatically mutated, IgG-secreting transgenic hybridomas (21, 22). All others are IgM-secreting transgenic hybridomas. The 378 base-protected VDJ fragment is indicated.

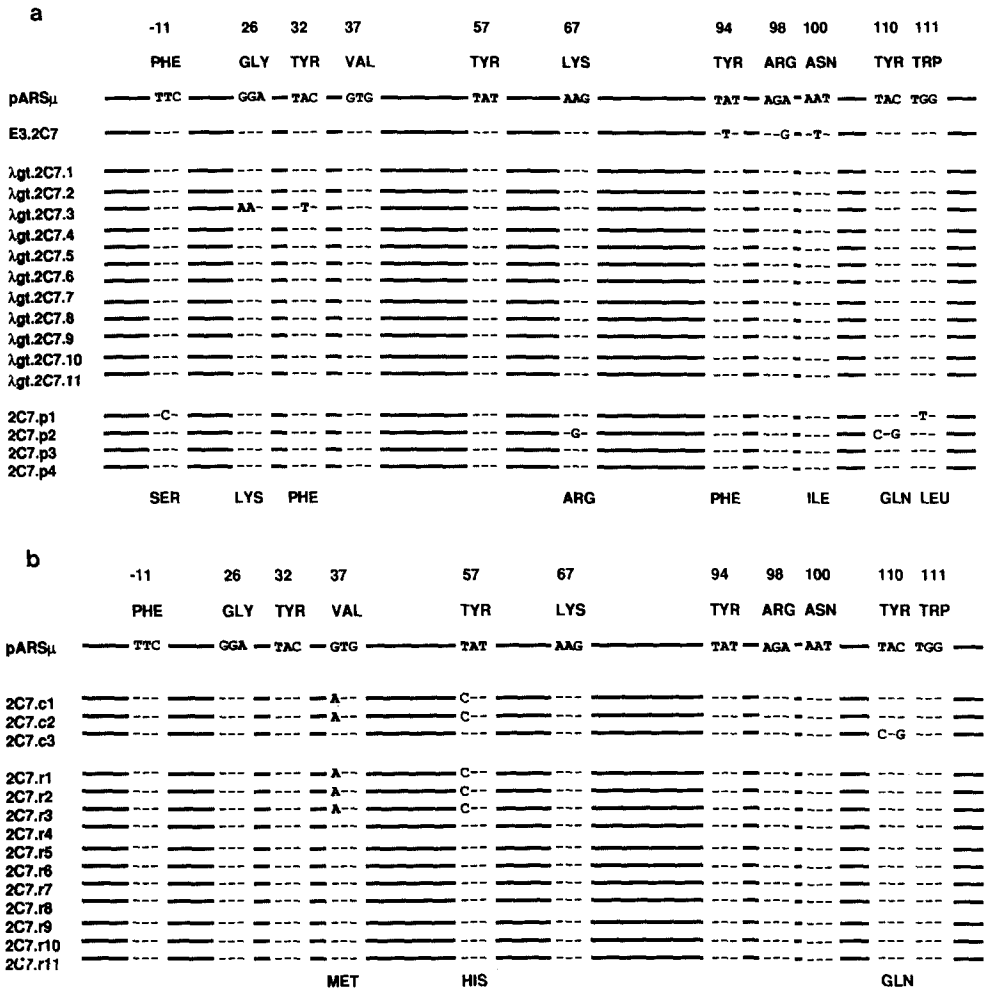


Figure 3. Comparison of transgene VDJ sequences found in IgM hybridoma 2C7 to ARSμ. Sequences identical to ARSμ (21) are indicated either by a solid line or by a dash within those codons that exhibit mutations. Position numbers of codons containing mutated bases are shown. Amino acids of ARSμ (*top*) and the replacement (*bottom*) at mutated codons are also indicated. (*a*) E3.2C7 is an EMBL3 clone, λgt.2C7.1–11 are λgt10 clones, both obtained from genomic libraries. 2C7.p1-p4 are genomic PCR clones. (*b*) 2C7.c1-c3 are λgt10 clones obtained from a 2C7 cDNA library, and 2C7.r1-r11 are PCR clones derived from 2C7 cDNA.

tions equally well, this approach does identify some mutated VDJ regions in expressed transgene copies.

RNAs from 16 IgM transgene-expressing hybridomas that were derived from immunized transgenic mice, were screened by RNase protection analysis (Fig. 2 *b*). Partially protected, smaller-sized bands were observed in 2C7, indicating the expression of mutated transgene copies in this cell line. The presence of strong full-length protected bands in 2C7 also suggested either expression of unmutated transgene copies, or expression of copies that had mutations that were not revealed by the RNase protection assay. Other hybridomas in Fig. 2 *b* (such as 2G8) displayed low levels of smaller-sized protection bands, but these were weak and shared by many of the cell lines. Thus, any suggestion of mutated copies in these hybridomas by the protection assay was marginal.

Mutations in the Transgene V Region in an IgM Hybridoma. To determine whether the transgene copies in 2C7 displayed somatic mutations, we sequenced transgene VDJ regions in 2C7 genomic DNA by two approaches. First, we PCR-amplified the transgene VDJ regions from 2C7 genomic DNA, isolated PCR clones, and sequenced individual clones. Among four of these 2C7 DNA PCR clones, two did not exhibit any mutations and two clones had two and three mutations, respectively, in the 470-bp VDJ region sequence (Fig. 3 *a*). We have determined that the error rate of Taq polymerase in our system is ~1/2,500 by sequencing 10 transgene VDJ region PCR clones (470 bp/clone) obtained from 30 cycles of PCR amplification of transgenic kidney DNA. This is six-fold less than the rate of mutation found in the PCR clones of the transgene V regions in 2C7. Thus, although the overall frequency of mutations in the 2C7 DNA PCR clones is low (1/390), our results suggest that some of these reflect genuine somatic mutations.

To investigate mutation of transgene copies in 2C7 using a second approach, EMBL3 and λgt10 genomic libraries of 2C7 DNA were constructed and the phage clones containing transgene VDJ sequences were isolated. One EMBL3 (E3.2C7) clone and eleven λgt10 (λgt.2C7.1–11) genomic clones that contained the transgene VDJ region were obtained. For E3.2C7, sequences of the VDJ region and portions of enhancer and C regions were determined. Three mutations were found in the 470-bp VDJ region (Fig. 3 *a*), whereas no mutations were found in the 600-bp enhancer and 350-bp C region sequences. 11 λgt10 clones were screened for mutations by DGGE after PCR amplification in the VDJ region covering CDR1 to CDR3. 10 of these showed no band shift upon

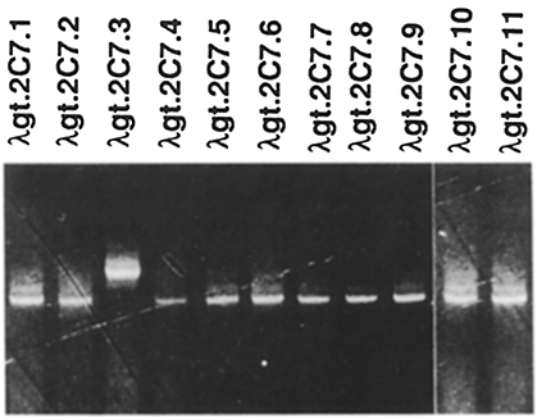


Figure 4. DGGE analysis of λgt2C7 clones. Each of eleven λgt2C7 genomic clones was PCR amplified and the PCR products were analyzed by denaturing gradient PAGE to screen for the presence of mutations.

DGGE and showed no mutations in subsequent sequence analyses. One clone, λgt.2C7.3, did show a band shift upon DGGE analysis (Fig. 4), suggesting the presence of mutation. Sequence analysis of λgt.2C7.3 demonstrated three mutations in the VDJ region (Fig. 3 *a*), but no mutations in the 600-bp enhancer region. Thus, analyses of these genomic clones confirmed that some μ transgene copies were mutated in the 2C7 hybridoma and indicated that transgene mutations were localized to the region surrounding the VDJ exon.

Expression of Mutated and Unmutated Transgene Copies in 2C7. To investigate whether mutated transgene copies in 2C7 were expressed at the RNA level we used two approaches to sequence the transgene VDJ regions present in 2C7 mRNA. First, three transgene cDNA clones (2C7.c1–c3) were derived from a 2C7 λgt10 cDNA library and sequences of the VDJ and CH1 regions were determined. As shown in Fig. 3 *b*, each of the three clones exhibits two mutations within the VDJ region. Two clones, 2C7.c1 and 2C7.c2 have identical mutations, one at codon 37 and the other at codon 57. The third cDNA clone, 2C7.c3, also has two mutations, both within codon 110. For the CH1 region, one mutation was found in 2C7.c1, whereas no mutations were found in 2C7.c2 and 2C7.c3.

Subsequently, we PCR-amplified transgene cDNA from 2C7 mRNA, isolated PCR clones, and sequenced the VDJ region of 11 cDNA PCR clones (2C7.c1–2C7.r11; Fig. 3 *b*). Among these, eight clones were unmutated, and three clones had two mutations each. Remarkably, each of the three mutated clones exhibited identical mutations (one at codon 37 and the other at 57) These mutations were the same as found in two of the three λgt10 cDNA clones, 2C7.c1 and 2C7.c2. Thus, this pattern of two mutations appears to be present at a high frequency among the transgene RNAs in the 2C7 hybridoma.

Our sequence analysis of clones derived from 2C7 DNA and RNA provides strong evidence for at least four mutated transgene copies in the 2C7 hybridoma. Two mutated copies are represented by the E3.2C7 and λgt.2C7.3 genomic clones, one mutated copy appears to be represented by several cDNA or cDNA PCR clones (e.g., 2C7.c1), and an additional mutated copy may be represented by a DNA PCR clone (2C7.p2) and a cDNA clone (2C7.c3). Although 2C7.p2 and 2C7.c3 differ by one nucleotide, these two clones do share two mutations and the single difference could reflect a PCR error.

Chromosomal Location of the 2C7 Transgene. We have previously shown that the ARSμ transgene can undergo interchromosomal DNA recombination resulting in the linkage of the transgene to the endogenous C_H locus (21, 22). Analysis of the IgM produced by the 2C7 hybridoma indicated expression of the μ^a allotype that is characteristic of the transgene Cμ, but no detectable expression of the endogenous μ^b allotype (data not shown). This suggests that the 2C7 transgene array has not recombined to the endogenous Ig H chain locus, but remains at the ARS5 chromosome 5 integration site determined previously (22). To confirm the chromosomal location of the transgene array in 2C7, we used in situ hybridization with chromosomal metaphase spreads under the conditions that we had previously found to yield detectable hybridization only to the multicopy transgene array (22). Only one site of hybridization proximal to the centromere of a single chromosome was found in 2C7 (Fig. 5 *a*); this pattern is analogous to our previous findings in normal ARS5 cells (22). Thus both serological and chromosomal analyses indicate that the transgene copies in 2C7 have not undergone chromosomal translocation. This demonstrates that sequences associated with the endogenous C_H region are not involved in mutation of the 2C7 transgene copies.

Transgene Mutations in a Second IgM Hybridoma. To explore whether transgene mutation in the 2C7 hybridoma represents a rare event, we analyzed the mutational status of a second IgM-producing hybridoma, 2G8. We chose to characterize 2G8 based on a marginal suggestion of mutations in our RNase protection assay (Fig. 2 *b*). Sequence analysis of 2G8 PCR clones obtained from either genomic DNA (Fig. 6 *a*) or cDNA (Fig. 6 *b*) demonstrated nucleotide changes in the transgene VDJ regions. As found for 2C7, the frequency of mutations in the 2G8 genomic DNA PCR clones was slightly elevated (threefold) above our background PCR error rate. More convincingly, mutations were also found in the 2G8 cDNA PCR clones and independent clones exhibited the same mutations. Two of the cDNA PCR clones, 2G8.a3 and 2G8.b7, which were derived from separate PCR reactions, exhibited identical patterns of four mutations. The probability that four random mutations in 390 bp of sequence would be exactly repeated among 15 clones is on the order of 10^{-11}. In addition, the cDNA PCR clones, 2G8.a2 and 2G8.b3, also derived from separate PCR reactions, each displayed the same two mutations which were also shared with the 2G8.a3 and 2G8.b7 clones (Fig. 6 *b*). Furthermore, the 2G8.b8 clone displayed the same four mutations as 2G8.a3 and 2G8.b7, but also had one additional nucleotide change (Fig. 6 *b*). Although we do not know the origin of these patterns of related but nonidentical changes between different cDNA PCR clones (see Discussion), the results clearly indicate the presence of at least one mutated and expressed transgene copy in 2G8.

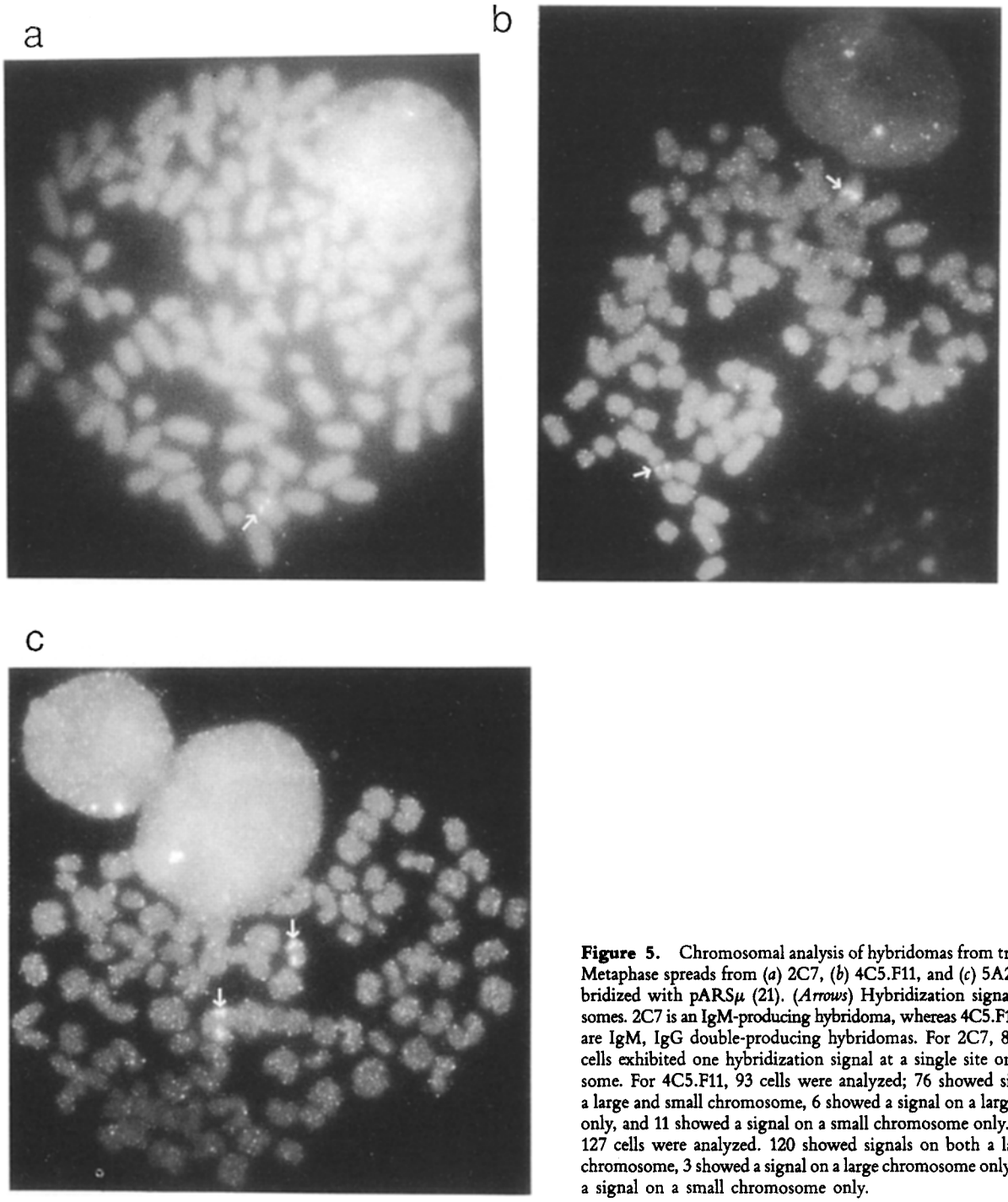


Figure 5. Chromosomal analysis of hybridomas from transgenic mice. Metaphase spreads from (*a*) 2C7, (*b*) 4C5.F11, and (*c*) 5A2.A11 were hybridized with pARSμ (21). (*Arrows*) Hybridization signals on chromosomes. 2C7 is an IgM-producing hybridoma, whereas 4C5.F11 and 5A2.A11 are IgM, IgG double-producing hybridomas. For 2C7, 87/89 analyzed cells exhibited one hybridization signal at a single site on one chromosome. For 4C5.F11, 93 cells were analyzed; 76 showed signals on both a large and small chromosome, 6 showed a signal on a large chromosome only, and 11 showed a signal on a small chromosome only. For 5A2.A11, 127 cells were analyzed. 120 showed signals on both a large and small chromosome, 3 showed a signal on a large chromosome only, and 4 showed a signal on a small chromosome only.

Successive Introduction of Transgene Mutations During B Cell Proliferation. As reported previously, the μ transgene in ARS5 transgenic mice can undergo transchromosomal isotype switch recombination with endogenous γ genes after antigenic stimulation (21, 22). We have found that, even after extensive subcloning, some hybridomas derived from immunized ARS5 transgenic mice express and secrete both IgM and IgG transgene products, as determined serologically, by Northern blot and PCR analyses, and by immunoprecipitation and fractionation of μ and γ chain proteins on reducing SDS polyacrylamide gels (data not shown). Two of these double-producing hybridomas were also analyzed by in situ hybridization. In both, chromosomal translocations of transgene copies similar to those described previously (22) were found (Fig. 5, *b* and *c*).

The VDJ sequences of the switched γ transgenes in nine double-producing hybridomas were determined. Five of these showed only 0–1 mutations in the γ transgene copy. However, hybridomas 4C5.F11 and 5B6.G11 each displayed seven

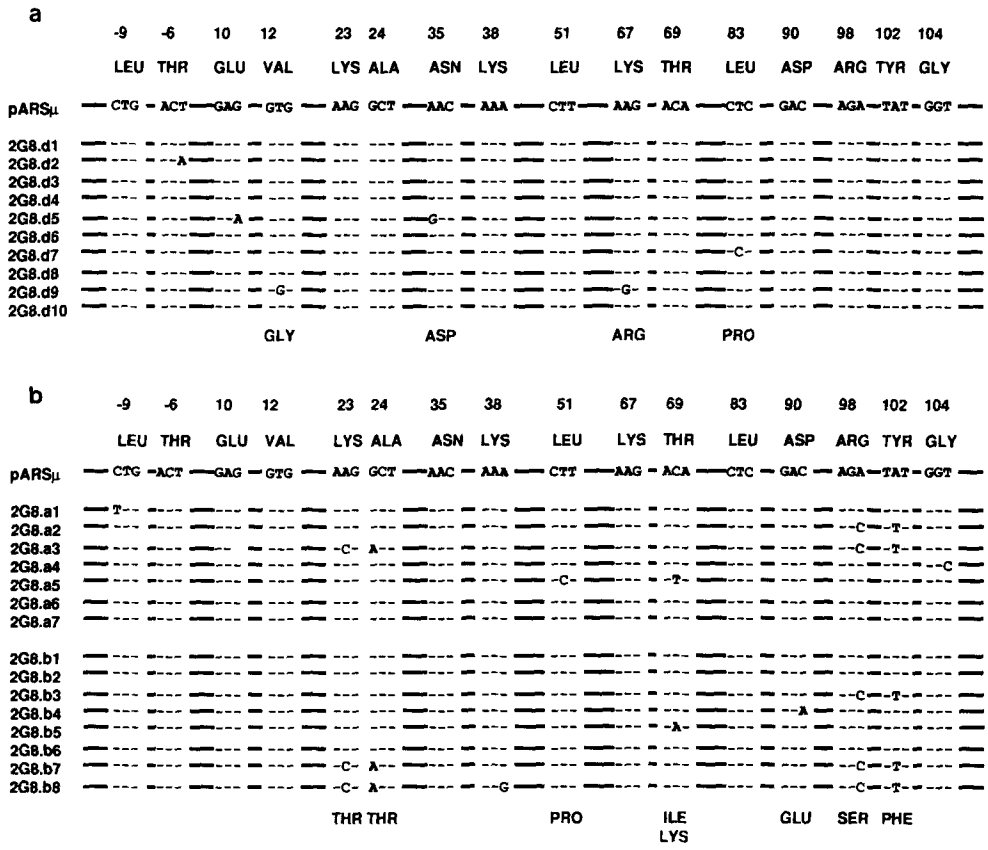


Figure 6. Comparison of transgene VDJ sequences found in the IgM hybridoma 2G8 to ARSμ. (*a*) 2G8.d1–d10 are genomic PCR clones. Sequences are compared as in Fig. 3. (*b*) 2G8.a1–a7 and 2G8.b1–b8 are independent sets of cDNA PCR clones derived from 2G8 mRNA.

mutations in the γ transgene copies, whereas hybridomas 5A2.A11 and 1G10.C4 each displayed eight transgene mutations. All of the detected mutations were established by sequencing two different PCR clones. Of the mutations found in the 4C5.F11 and 5B6.G11 hybridomas, five are identical in the two cell lines, whereas in each hybridoma two additional mutations are unique (Fig. 7). Further analyses showed that 4C5.F11 and 5B6.G11 shared recombined endogenous JH alleles as determined by Southern blotting; that both hybridomas expressed somatically mutated versions of the C57BL/6 Vκ10 L chain gene; and that one somatic mutation in the expressed Vκ10 genes was shared by the two hybridomas (among three Vκ10 mutations in 5B6.G11 and two Vκ10 mutations in 4C5.F11; data not shown). Because the 4C5.F11 and 5B6.G11 hybridomas were derived from a single immunized transgenic mouse, our data indicate that the two hybridomas represent fusions of separate B cells that were derived from clonal expansion of a common precursor in vivo. These results imply that transgene hypermutation can occur in stepwise fashion during in vivo proliferation of antigen-stimulated B cells. Analogous findings have been previously reported for endogenous Ig genes (33–36). Thus, our observations emphasize the similarity of somatic hypermutation for endogenous and transgenic Ig genes.

Higher Levels of Mutation in γ Transgene Copies Among μ, γ Double-producing Transgenic Hybridomas. Previous reports have shown that productive and nonproductive V(D)J recombined alleles in normal B cells exhibit similar levels of somatic hypermutation (12–14). The μ and γ double-producing hybridomas that we characterized afforded an opportunity to compare directly the level of mutation in μ and γ H chain transgenes present within a single cell. To focus on B cells that had clearly undergone extensive somatic hypermutation, we analyzed the three double-producers, 4C5.F11, 5A2.A11, and 1G10.C4, which exhibited the largest numbers of mutations in the γ transgene copies. Mutations in the μ transgene copies were studied by sequencing PCR clones of μ cDNA derived from each hybridoma. As shown in Table 1, in eight PCR clones of the μ transgene VDJ from each cell line, a total of eight, five, and three mutations were found in 4C5.F11, 5A2.A11, and 1G10.C4, respectively. Thus, the average number of mutations in μ transgene copies (16 mutations/24 copies = 0.7) appears to be 10-fold lower than the number found in the γ transgene copy present within the same cell (23 mutations/3 copies = 7.7). This 10-fold difference in the frequency of mutation is likely to be an underestimate due to the background of PCR errors in μ transgene PCR clones. Our results clearly indicate that the μ and γ transgene copies in these hybridomas do not show equivalent levels of hypermutation.

Discussion

Our studies indicate that an Ig μ H chain transgene can undergo somatic hypermutation without being linked to the endogenous Ig locus and, therefore, that the 15-kb transgene, which contains the VDJ and Cμ exons as well as surrounding

Table 1. *Transgene VDJ Sequences in IgM, IgG-producing Hybridomas*

Cell line	Isotype	Clone no.	No. of mutations	Position and type of mutation		
				Ser Asn	Ile Leu	Tyr Phe
4C5.F11	γ2b		7	31(AG*C→AA*C)	34(A*TA→C*TA)	57(TA*T→TT*T)
				Lys Ser	Tyr Asp	Thr Thr
				59(AA*G*→AG*C*)	80(T*AC→G*AC)	87(ACA*→ACG*)
				Ala Ser		
	μ	a1	1	97(G*CA→T*CA)		
				Phe Ser		
	μ	a2	1	64(TT*C→TC*C)		
				Ser Pro		
	μ	a3	1	21(T*CC→C*CC)		
	μ	a4	0			
				Thr Thr	Thr Thr	Ala Ser
	μ	b1	3	71(ACT*→ACC*)	78(ACA*→ACT*)	97(G*CA→T*CA)
				Val Ala		
	μ	b2	1	56(GT*T→GC*T)		
				Gln stop		
	μ	b3	1	5(C*AG→T*AG)		
	μ	b4	0			
				Gln Gln	Glu Asp	Phe Leu
5A2.A11	γ2b		8	3(CAG*→CAA*)	46(GAA*→GAT*)	56(G*TT→C*TT)
				Lys Ile	Lys Arg	Glu Glu
				59(AA*G*→AT*A*)	67(AA*G→AG*G)	89(GAG*→GAA*)
				Ala Ser		
				92(G*CA→T*CA)		
				Tyr Ser		
	μ	a5	1	102(TA*T→TC*T)		
	μ	a6	0			
	μ	a7	0			
	μ	a8	0			
				Val Ala	Ala Ala	
	μ	b5	2	−7(GT*A→GC*A)	92(GCA*→GCG*)	
				Tyr Ser		
	μ	b6	1	102(TA*T→TC*T)		
				Ile Val		
	μ	b7	1	48(A*TT→G*TT)		
	μ	b8	0			
				Phe Cys	Leu Leu	Pro Thr
1G10.C4	γ2a		8	−13(TT*T→TG*T)	51(CTT*→CTC*)	53(C*CT→A*CT)
				Thr Ser	Lys Ile	Ile Ala
				58(AC*T→AG*T)	59(AA*G*→AT*C*)	69(A*CA→G*CA)
				Phe Tyr		
				108(TT*T)→TA*T)		

continued

Table 1. *(continued)*

Cell line	Isotype	Clone no.	No. of mutations	Position and type of mutation
				Ser Thr Ser Asn
	μ	a9	2	31(AG*C→AC*C) 105(AG*C→AA*C)
				His His
	μ	a10	1	−2(CAC*→CAT*)
	μ	a11	0	
	μ	a12	0	
	μ	b9	0	
	μ	b10	0	
	μ	b11	0	
	μ	b12	0	

* Indicates nucleotides changed.

sequences, contains the sequence elements required for targeting the hypermutational mechanism to the H chain VDJ region. This is the first localization of sequences required for H chain hypermutation. Analogous studies have localized sequences important for hypermutation of L chain transgenes to a region of similar size (18–20).

Development of rapid and sensitive screening methods for point mutations is likely to be important in analyses of somatic mutation in transgenic mice. We have used both RNase protection and DGGE as screening methods to detect point mutations in transgene VDJ regions. RNase protection is not able to detect all single base substitutions (29, 31, 32), however, this assay enabled us to find IgM hybridomas that exhibit mutations among 30–50 μ transgene copies. We also used DGGE to screen transgene clones for mutations. It has been indicated that DGGE can detect over 95% of single base substitution mutations (27, 28). Our DGGE analyses of transgene clones illustrate the sensitivity of this assay because the observed mobility shift for the λgt.2C7.3 clone was actually due to a single A to T substitution.

Microinjected transgenes occasionally exhibit rare mutations that may result from the chromosomal integration process (37). However, it is unlikely that the mutations we have found in μ transgene copies represent such events. Mutations introduced into transgene copies during microinjection will be present in all cells and would be found repeatedly among different hybridomas. Yet, among 122 clones of the transgene VDJ region in ARS5 mice that have been sequenced, we have not found any examples of mutated transgene copies that repeat between different hybridomas. Furthermore, our RNAase protection experiments indicate that the 2C7 hybridoma expresses a mutated transgene copy that is not expressed in other hybridomas, and our sequence analysis of two transgene copies isolated from 2C7 shows the localization of mutation in the transgene VDJ region. Taken together, these results strongly support our conclusion that the transgene mutations are introduced by a somatic hypermutational process.

Several characteristics of the transgene mutations that we have detected indicate that the normal Ig gene hypermutational process is involved in transgene mutation. First, all transgene mutations found in this study are base substitutions. In normal Ig genes, over 95% of the mutations are also base substitutions (9, 14, 38). Second, mutations in the transgene appear to be localized largely to the VDJ region, consistent with findings for normal Ig genes (7, 8, 39). We did find one mutation in the CH1 exon of 2C7.c1. This mutation could represent an error of reverse transcriptase during cDNA synthesis (40) or a bona fide somatic mutation. Supporting the latter possibility, low levels of mutation in C region genes have been reported for Cλ genes in normal mouse hybridomas (14). Finally, two hybridomas isolated from a single immunized animal show a pattern of shared transgene mutations that indicates sequential mutation during cell proliferation. This is the first demonstration that Ig transgene mutations accumulate through several cell divisions. A number

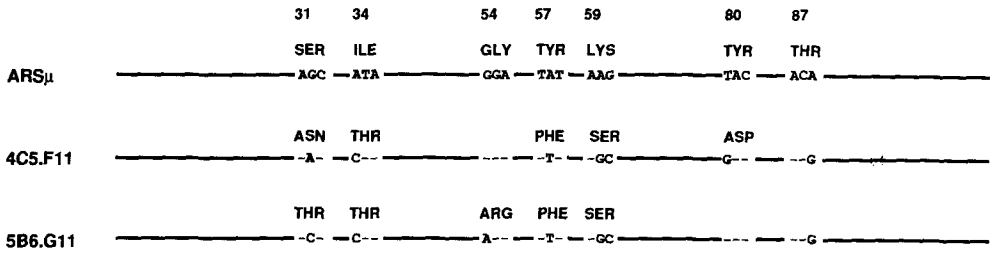


Figure 7. Comparison of VDJ sequences of γ transgene copies in 4C5.F11 and 5B6.G11 to ARSμ. Sequences are compared as in Fig. 3.

of reports have described similar results for endogenous Ig genes (33–36).

Sequence analyses of transgene VDJ regions from both DNA and RNA of the IgM hybridomas show that multiple, but not all, transgene copies have undergone mutation and at least some of the mutated copies are transcribed. It is interesting that our results suggest that some transgene copies might be overrepresented among transgene RNAs in a single cell. In 2C7, 5 out of 14 transgene cDNA-derived clones (36%) have the same mutations, i.e., a G to A substitution at codon 37 and a T to C substitution at codon 57. These mutations were not found in any of the 16 transgene clones obtained from 2C7 genomic DNA, suggesting that the majority of the transgene copies do not have those particular mutations. Similarly, in 2G8, 5 out of 15 cDNA PCR clones (33%) display an A to C mutation at codon 98 and an A to T mutation at codon 102, but none of the 10 genomic DNA PCR clones have these mutations. The pattern of mutations among 2G8 cDNA PCR clones is unusual. 2G8.a2 and 2G8.b3 share mutations at codons 98 and 102. These two mutations are also observed in three other clones (2G8.a3, 2G8.b7, and 2G8.b8), each of which share two additional mutations at codons 23 and 24. 2G8.b8 has a further A to G substitution at codon 38. This pattern of nested mutations among these clones could reflect PCR errors of "template jumping" (41) and base misincorporation (42). However, we cannot rule out that these results might reflect a role for gene conversion in transgene mutation.

Our results indicate that several mutated transgene copies, as well as unmutated copies, can be found within a single cell. Sharp et al. (20) have also found that multiple κ transgene copies can be mutated within a single B cell and that mutated and unmutated copies can be found in the same cell. On the other hand, Rogerson et al. (43) have noted that, in one κ transgenic mouse line, only one of three κ transgene copies within a single B cell shows high levels of somatic mutation (although the particular transgene copy that is mutated can vary between different B cells) and have suggested a role for DNA replication in somatic hypermutation. We do not know why some μ transgene copies are not mutated in B cells that have clearly undergone the hypermutational process. Possibly this reflects a process, such as DNA replication, that might regulate the hypermutational mechanism. However, because the number of mutations per μ transgene copy that we have found is relatively low, it also seems possible that the presence of mutated and unmutated μ transgene copies in a single cell could be merely a statistical phenomenon.

Our studies of transgene mutation in IgM and IgG double-producing hybridomas are the first to assess somatic hypermutation of μ and γ genes within a single cell. We find that in three hybridomas, μ transgene copies exhibit at least 10-fold fewer mutations than the isotype-switched γ transgene copy present in the same cell. These results are intriguing because previous reports have indicated that productive and nonproductive V(D)J joined genes in a single cell exhibit very similar levels of somatic mutation (12–14). There are likely to be many plausible models which could account for the nonequivalent hypermutation of μ and γ transgene copies that we observe. Because we find that several different μ transgene copies can be mutated in an IgM-producing cell, we suspect that the higher levels of mutation observed in γ transgene copies might be related to isotype switching. Increased mutation of γ transgene copies could indicate that sequences located within or near the Cγ locus (perhaps even the enhancer sequence located 3′ of the Cα gene [44]) might enhance hypermutation of a transgene VDJ region which is juxtaposed to the endogenous Cγ region by isotype switching. Such an effect would presumably also occur with endogenous H chain VDJ regions and might account for the larger numbers of mutations that are often found in IgG- versus IgM-producing B cells in nontransgenic mice (39, 45, 46). There is also a precedent for the notion that sequences downstream of the antibody V(D)J region might enhance the frequency of hypermutation (19, 20).

The higher levels of γ transgene mutation that we have found in double-producing B cells could potentially also be explained by effects that result from antigenic selection. This hypothesis requires several assumptions. First, because productive and nonproductive alleles are mutated at equivalent levels in normal B cells (12–14), it is clear that normal somatic hypermutation rates are so high that silent mutations are coselected with mutations that affect antigen binding. This would suggest that antigenic selection should not focus mutations in the γ transgene copy within transgenic B cells that express both IgM and IgG. However, if the somatic mutation rate per gene is lower than normal in multicopy transgenic B cells, then it could be suggested that during cell proliferation mutations in the γ transgene copy might be preferentially selected, whereas mutations in μ transgene copies might be diluted. Preferential antigenic selection of mutations in the γ transgene copy could be based on a more effective signal transduction for proliferation through surface IgG versus surface IgM. As mentioned, this model relies on the assumption of a decreased mutation rate per gene in multicopy transgenic B cells. However, previous work has indicated that the frequency of mutations per transgene copy in κ transgenic mice appears to be unaffected by copy number (18, 20). Nevertheless, because previous studies have used transgenic mice carrying between 3 and 10 transgene copies, it is possible that a decreased mutation rate occurs in cells that have higher copy numbers.

A recent report showing no somatic hypermutation of a TCR transgene expressed in B cells under the control of the Ig H chain enhancer indicates that the H chain enhancer sequence is irrelevant or insufficient for targeting transgene hypermutation (37). Our finding that hypermutational targeting can be directed by a 15-kb μ transgene indicates that deletional mutagenesis of H chain transgene constructs can lead to the identification and characterization of those *cis*-acting sequences that are required by the mutational machinery. It will also be interesting to compare transgenes encoding μ and γ H chains to determine whether sequences that enhance mutation might be localized near the Cγ exons.

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