Comparison of Identical and Functional $IgH$ Alleles Reveals a Nonessential Role for Eμ in Somatic Hypermutation and Class-Switch Recombination

Fubin Li, Yi Yan, Joyce Pieretti, Danielle A. Feldman and Laurel A. Eckhardt

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Comparison of Identical and Functional Igh Alleles Reveals a Nonessential Role for Ep in Somatic Hypermutation and Class-Switch Recombination

Fubin Li, Yi Yan, Joyce Pieretti, Danielle A. Feldman, and Laurel A. Eckhardt

Somatic hypermutation (SHM), coupled with Ag selection, provides a mechanism for generating Abs with high affinity for invading pathogens. Class-switch recombination (CSR) ensures that these Abs attain pathogen-appropriate effector functions. Although the enzyme critical to both processes, activation-induced cytidine deaminase, has been identified, it remains unclear which cis-elements within the Igh loci are responsible for recruiting activation-induced cytidine deaminase and promoting its activity. Studies showed that Igh gene-transcription levels are positively correlated with the frequency of SHM and CSR, making the intronic, transcriptional enhancer Ep a likely contributor to both processes. Tests of this hypothesis yielded mixed results arising, in part, from the difficulty in studying B cell function in mice devoid of Ep. In Ep’s absence, VH gene assembly is dramatically impaired, arresting B cell development. The current study circumvented this problem by modifying the murine Igh locus through simultaneous insertion of a fully assembled VH gene and deletion of Ep. The behavior of this allele was compared with that of a matched allele carrying the same VH gene but with Ep intact. Although Igh transcription was as great or greater on the Ep-deficient allele, CSR and SHM were consistently, but modestly, reduced relative to the allele in which Ep remained intact. We conclude that Ep contributes to, but is not essential for, these complex processes and that its contribution is not as a transcriptional enhancer but, rather, is at the level of recruitment and/or activation of the SHM/CSR machinery. The Journal of Immunology, 2010, 185: 6049–6057.

During an immune response, somatic hypermutation (SHM) introduces point mutations into the Igh genes of germinal center B cells at a rate 1 million-fold greater than that of spontaneous mutation (reviewed in Refs. 1–3). SHM’s role in Ab diversity takes place after Ag stimulation of individual B cells, contributing to these cells’ developing Abs with increasing affinity for the Ag.

The biochemical mechanism of SHM has been subjected to intensive study, and the discovery of activation-induced cytidine deaminase (AID) and other important players has greatly improved our understanding of this process. AID activity is also required for the process of class-switch recombination (CSR) (4). Although the efficiency of an Ab response is greatly augmented by SHM and CSR, mistargeting of these processes poses a significant threat to the integrity of the host cell’s genome. In fact, AID has been implicated in tumorigenesis in a wide variety of tissue types (5, 6). Fortunately, AID normally acts almost exclusively on Igh genes. Although it was shown that some non-Igh genes are subject to AID-induced cytidine deamination (in B cells of normal mice and healthy humans), repair of these lesions is such that no mutation results or mutation occurs at rates at least an order of magnitude less than that at Igh loci (7–11).

The means by which the SHM machinery is specifically directed to Igh loci remains unclear (reviewed in Refs. 1–3). SHM preferentially targets RGYW/WRCY hot spots, but these hotspots can be found in almost any gene. Because VH sequences can be replaced with other DNA sequences without affecting the rate or extent of SHM, element(s) responsible for targeting must fall outside the region mutated but in cis with those sequences (12). A strong promoter is required, but while an Igh promoter is not essential, not all other strong promoters will suffice (13, 14). The tissue-specific transcriptional enhancers associated with the Igh loci have been implicated, but their importance in transgenes has not always been matched by an equivalent importance within the endogenous loci (15–19). Furthermore, because transcription rate is positively correlated with SHM rate (13, 20), it is difficult to parse whether enhancers serve to mark a locus for mutation (i.e., recruit the SHM machinery) and/or increase the rate of mutation by simply increasing transcription (i.e., influence machinery activity subsequent to recruitment).

Enhancers have also been implicated in the control of CSR. Deletions affecting the 3’ regulatory region (3’RR) of the Igh locus dramatically reduce CSR (21–24). An important role was
attributed to the intrinsic enhancer Eμ, when it was found that deletion of the core (cEμ) or the core and flanking matrix attachment regions (MARs) reduced CSR activity (25, 26). However, in these early experiments, cEμ’s essential role in VH gene assembly precluded CSR analyses of functional IgH genes; Eμ-deficient alleles were uniformly nonfunctional (no VDJ assembly) and were assessed for CSR only by assays for DNA rearrangements in and around the switch-μ (Spμ) region (27–30). In later studies, germline transmission of a cEμ-deficient allele allowed for the analysis of rare cEμΔ/CεμΔ B cells that had undergone monoallelic VH gene assembly in cEμ’s absence (29). As before, the nonfunctional, cEμ-deficient allele in these rare cells showed evidence of reduced CSR activity (measured by Spμ breaks/ rearrangements). CSR and SHM took place on the functional (but cEμ-deficient) allele, but it was not possible to compare the rates of these processes with those on an allele that retained cEμ, because, when made heterozygous with a wild-type (WT) allele, the cEμ-deficient allele never became functional (no V–D–J assembly). Another caveat to assessing the effect of cEμ deletion on CSR and SHM in cEμΔ/CεμΔ mice was the possibility that the rare B-lineage cells that managed to form a functional IgH gene might have done so through compensatory mutations that supplanted Eμ function with respect to both VH assembly and SHM/CSR.

In this study, we sought to circumvent the fundamental problem associated with studying Eμ’s functions in processes that take place after VH gene assembly. The strategy taken was to generate and then directly compare matched IgH alleles that carried identical VH genes (VH knock-in) but retained Eμ (VH/Eμ) or lacked Eμ (VHΔ). As described below, this comparison revealed a reduction in CSR and SHM upon Eμ deletion, but the effects observed were small and suggest considerable functional redundancy in the locus with respect to the regulation of these processes. Given that loss of Eμ did not result in decreased IgH gene transcription in mature, activated B cells, we further suggest that Eμ’s role in SHM and CSR is not as a regulator of transcription but, rather, as one of the elements contributing to the recruitment of the SHM/CSR machinery to the IgH locus.

Materials and Methods

Mice

VHΔ mice on a mixed C57BL/6J and 129/Ola genetic background were generated in our laboratory (31) and later backcrossed to mice with a C57BL/6J genetic background. Homozygous VH/Eμ mice on a mixed C57BL/6 and 129/Ola genetic background were previously described in B1-8i mice (32) and were kindly supplied by Dr. Klaus Rajewsky (CNR Institute for Biomedical Research, Boston, MA). Mice carrying IgH alleles that lacked the JH gene segments and Eμ (denoted ΔEμ in the present studies) were on a C57BL/6J genetic background and were obtained from The Jackson Laboratory (Bar Harbor, ME) (B6.129P2-Igh-Jtm1Cgn/J; stock no. 74023) (28). All mice were bred and maintained in animal facilities at Hunter College, City University of New York, in accordance with established guidelines of the Hunter College Institutional Animal Care and Use Committee.

shm analyses

Germinal center B cells from Peyer’s patch. Germinal center B cells were isolated as B220+CD95+cεμ-D NA mice expressed from the VHE allele; primers no. 4 and no. 2 specifically amplified this region from the VHE allele (Fig. 1A). PCR products were cloned with high-fidelity PfuUltra Hotstart DNA polymerase (cat. no. 600300; Stratagene, La Jolla, CA), and products were cloned in pCR4Blunt-TOPO vector (cat. no. K2880-20; Invitrogen, Carlsbad, CA) for sequencing. Included in these studies were VH186.2Δ/CεμΔ mice (4), and VHE/VμΔ mice.

Splenic germinal center B cells. Mice were immunized once with 4-hydroxy-3-nitrophenylacetyl chicken γ globulin (NP-CGG) or SRBCs and sacrificed 7 d later. Germinal center B cells were isolated as B220+CD95+ PNAhigh cells from one VHE/VμΔ mouse immunized with SRBCs; IgG+ germinal center B cells were sorted as B220+CD95+ IgG+ cells from three VHE/VμΔ mice immunized with SRBCs and two VHE/VμΔ mice immunized with NP-CGG in alum.

VH186.2Δ cDNA sequences. Three WT mice, four VHEΔ/WT mice, and three VHE/Eμ mice were injected i.p. with 100 μg NP-CGG (Biological Technologies, Novato, CA; cat. no. N-5055) suspended in alum (Pierce, Rockford, IL; cat. no. 77161), according to the manufacturer’s instructions. Mice were boosted once or twice at 3-wk intervals. One week after the last injection, spleens were harvested, and RNA was extracted using a Qiagen RNeasy mini kit (Qiagen, Valencia, CA; cat. no. 74104). VH186.2Δ cDNAs were amplified by RT-PCR using a Qiagen one-step RT-PCR kit (Qiagen; cat. no. 210212) with primers VH186.2F and Cy1R, described previously (4). VH186.2F anneals to VH186.2 leader sequence, and Cy1R anneals to CH1 of Cy, amplifying VH186.2Δ transcripts by both IgL alleles in VHE/WT and VHEΔ/WT B cells. WT allele transcripts were easily distinguished from those derived from the mutant alleles through analysis of the VDJ junction (unique sequence for B1-8 VH) and presence/absence of a homology region (27–30). In later studies, primers VH186.2F and VH186.2R anneals to CH1 of C, amplifying VH186.2Δ transcripts by both IgL alleles in VHE/WT and VHEΔ/WT B cells. WT allele transcripts were easily distinguished from those derived from the mutant alleles through analysis of the VDJ junction (unique sequence for B1-8 VH) and presence/absence of a homology region (27–30).

The JH in the present context is the JH gene segment that is deleted in the cEμΔ gene (VHE), and VH186.2Δ-CαμΔ/CεμΔ (within the 3’ homology region of B1-8i) was amplified by the manufacturer (User bulletin no. 2; http://docs.appliedbiosystems.com/life_science). Time PCR was performed using a Qiagen RNeasy mini kit (Qiagen, Valencia, CA; cat. no. 74104) from resting splenic B cells (enriched by negative selection using a B cell isolation kit from Miltenyi Biotec, Bergisch Gladbach, Germany; cat. no. 130-090-862), splenic B cells stimulated by LPS for 48 h, or germinal center cells isolated from Peyer’s patch by the TaqMan, one-step RT-PCR kit (Applied Biosystems, Foster City, CA; cat. no. 4309169, TaqMan gene expression assay ID for hprt1).

Total RNA was isolated with the RNeasy mini-prep kit (Qiagen; cat. no. 74104) from resting splenic B cells (enriched by negative selection using a B cell isolation kit from Miltenyi Biotec, Bergisch Gladbach, Germany; cat. no. 130-090-862), splenic B cells stimulated by LPS for 48 h, or germinal center cells isolated from Peyer’s patch by the TaqMan, one-step RT-PCR kit (Applied Biosystems, Foster City, CA; cat. no. 4309169, TaqMan gene expression assay ID for hprt1).

Analysis of DNA sequences. For sequences cloned from germinal center B cells, point mutations were analyzed in the 360-bp VH186.2-DFL16.1–Iγ2 coding sequences (exon) and the following 214-bp intron sequences (Fig. 1A). Total and synonymous mutations were identified in protein-encoding sequences (65.67 synonymous sites in V186.2 and 79.33 in VH186.2-DFL16.1–Iγ2). Synonymous sites were calculated with the Synonymous Non-synonymous Analysis Program (33). For V186.2Δ cDNA sequences, only point mutations observed in the V186.2 region were analyzed, and the V-Quest program (available at http://imgt.cines.fr) was used to analyze these sequences to identify the VH gene and mutations (34).

Real-time RT-PCR

Real-time RT-PCR analyses of VDJ-μ and VDJ-α mRNA expressed from the VHE and VHEΔ alleles were performed with the Qiagen QuantiTect SYBR Green RT-PCR kit (Qiagen; cat. no. 204243), using a 5’ primer specific for B1-8 VH (JH1 primer: 5’-CGCAAAGTACGACTTA CGG-3’), anneals to VDJ junction primer 1, amplifying VH186.2Δ transcripts (primer sequences available upon request). RNA amounts were normalized with hprt1 mRNA and were analyzed by the TaqMan, one-step RT-PCR kit (Applied Biosystems, Foster City, CA; cat. no. 4309169, TaqMan gene expression assay ID for hprt1).

Transcription rate comparisons by nuclear run-on assay

Methods used were essentially as described in Ref. 35. Splenic B cells from four VHE/Eμ mice and from four VHE/VμΔ mice were stimulated in culture with LPS (25 μg/ml) for 3 d. Cells were harvested, and nuclei were

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isolated and incubated with 500 μCi [α-32P]UTP (6000 Ci/mmol, 10 mCi/ml) for 30 min. Isolated RNA (RNEasy mini-prep kit; Qiagen; cat. no. 74104) was hybridized to dot blots of the following plasmids (5 μg each): plasmid vector (negative control, pBluescript II SK+), mouse actin cDNA (positive control), TOPO-VH (B1–8VH coding sequence), and TOPO-intron (the 214-bp sequence downstream of B1–8VH). Hybridization was performed for 4 d with 2-6×10^6 cpm RNA in a total volume of 1 ml (Ultra-hyb, Applied Biosystems; cat. no. AM8670). Blots were developed on a Typhoon 9410 PhosphorImager (GE Healthcare, Piscataway, NJ), and dot intensities were quantified with ImageQuant software.

**ELISA**

Sera prepared from 3–11-mo-old mice (4 WT; 4 VHE/B1–8VH/ΔJH; 5 VHE/ΔJH/ΔJc; 6 homozygous VHE/B1–8VH/ΔJc; 6 homozygous VHE/ΔJH/ΔJc mice) were analyzed for Ig isotype levels using a sandwich ELISA and standard methods. p-Nitrophenyl phosphate was used as substrate with alkaline phosphatase-conjugated Abs; ABTS was used as substrate for HRP-conjugated Abs.

Abs for the IgM, IgG1, IgG3, and IgE assays were purchased from BD PharMingen (San Diego, CA), IgG2a and IgG2b assays were carried out with Zymed Abs (Invitrogen), and the IgG2b assay was carried out with Abs from Meloy (Springfield, VA) (detailed information on Abs available upon request).

**Flow cytometry**

Generally, cells were incubated at 4°C for 15 min in staining buffer (PBS, 5.6 mM glucose, 0.1% BSA, 0.1% NaN3) with mAbs obtained from BD PharMingen, except where indicated. FITC-conjugated reagents included Abs to mouse (IgM, IgG3, IgG2a, IgG2b, IgG3, and IgE) and FITC-streptavidin. FITC-PNA was from Vector Laboratories (Burlingame, CA). PE-conjugated reagents included Abs to mouse (IgG1 and CD95) and PE-streptavidin. PE-anti-mouse IgM was from eBioscience (San Diego, CA). Additional reagents were allophycocyanin-conjugated anti-mouse B220 and biotin-conjugated anti-IgG2a and anti-IgG2b. Cells were analyzed using FACS instruments (FACScan, FACSCalibur, and FACS-Vantage; BD Biosciences, San Jose, CA). Side and forward scatter were used to gate on lymphocytes, and dead cells were excluded by propidium iodide staining. All sorting was done using FACSVantage. Data were acquired with CellQuest or Pro CellQuest (FACS instruments) and then further analyzed with FlowJo software (Tree Star, Ashland, OR).

**In vitro stimulation of splenic B cells and analysis**

Resting splenic B cells were enriched by negative selection (Miltenyi Biotec; cat. #130-090-862). Experiments were performed in parallel on cells isolated from WT, VHE/B1–8VH, VHE/ΔJH/ΔJc, and VHE/ΔJH/ΔJc mice. Cells were cultured for 3–5 d at 5×10^5 cells/ml in RPMI 1640 media supplemented with 20% FCS, 50 μM 2-ME, and 25 μg/ml LPS (Sigma-Aldrich, St. Louis, MO; cat. no. L6511), with or without the addition of cytokines: 10 ng/ml IL-4 (PeproTech, Rocky Hill, NJ; cat. no. 214-14), 100 ng/ml IFN-γ (PeproTech; cat. no. 315-05), or 1 ng/ml TGF-β (PeproTech; cat. no. 100-21R).

For CSR analyses shown in Fig. 3B, B cells were cultured for 5 d with LPS to stimulate switching to IgG3 and IgG2b, for 5 d with LPS + IL4 for switching to IgG1 and IgE, and for 3 d with LPS + IFN-γ for switching to IgG2a, and for 3 d with LPS + TGF-β for switching to IgG2a.

For the stimulation of splenic μHμH double producers shown in Fig. 4A and 4B, splenic μHμH cells were isolated from VHE/ΔJH/WT mice by flow cytometry) to a purity >98%. For assays of allele-specific class switching, cells were plated in culture with LPS (25 μg/ml), BAFF (10 ng/ml), and IFN-γ (100 ng/ml) for 5 d to induce switching to IgG2a, and cells were analyzed for the expression of IgG2a and IgG2b, by using ECL western blot analysis. B cells were incubated in these experiments for IgG2a and IgG2b allele controls, respectively. Alternatively, stimulated B cells were analyzed for surface IgM and IgM′ 3 d after stimulation with LPS + IFN-γ and 5 d after stimulation with LPS + IL4.

For DNA analyses of VHE/B1–8VH B cells, enriched B cells were stimulated with LPS for 4 d, and B220.IgG3+ or B220, IgG3− cells were isolated by flow cytometry. DNA was extracted from the sorted cells and analyzed by Southern blot for loss of a BamHI–EcoRI fragment that spans an intron (the 214-bp sequence downstream of B1–8VH). Hybridization was performed with a phosphorImager (Amersham Biosciences, Piscataway, NJ), and dot intensities were quantified with ImageQuant software.

**Results**

We used gene targeting to insert a fully assembled VΗ gene, VΗ/B1–8, into its natural position upstream of Cμ, while removing Eμ (Fig. 1A) (31, 32). The VΗ knock-in allowed us to circumvent the contribution to VΗ assembly (and B cell development) normally induced by Eμ removal (27–30). Mice heterozygous or homozygous for this modified IgH allele (designated VΗ/Δ) had normal numbers of B cells in bone marrow, spleen, and Peyer's patches compared with a matched mouse strain with VΗ knock-in but Eμ intact (allele designated VΗ/Eμ) (31, 32, data not shown). Given that the VΗ/Eμ and VΗ/Δ alleles carried the same VΗ promoter, VΗ sequence, and the same regulatory elements except for Eμ, a comparison of SHM and CSR in these two strains constituted a direct test of the effect of Eμ deletion on both.

**FIGURE 1.** WT and mutant IgH alleles and effect of Eμ deletion on SHM. A, Diagrams of the WT allele, B1–8VΗ with intact allelic (VΗ/Eμ) and B1–8VΗ knock-in allele with Eμ removed (VΗ/Δ). Coding exons (DS12, H2, B1–8VΗ, and Cα) are shown as filled boxes. Numbered arrows show the position and direction of primers used to clone sequences by PCR. Iμ, transcription start site for sterile Cα transcripts; arrowhead ends at donor splice site of noncoding exon (splice site indicated with curved line on VΗ/Δ map). Sμ, region of DNA breaks during CSR. Exon (360 bp) and Intron (214 bp) denote regions sequenced. The “intron” derives from downstream of JH2 (the JΗ segment used in B1–8VΗ). B, Pie charts comparing distribution of mutations among clones of VΗ (exon) and intronic (intron) sequences. Number at the center of each pie chart represents the total clones analyzed. Color legend indicates number of mutations per clone. Pie section percentages are provided in some cases (periphery of circle). Sequences cloned from Peyer’s patch germinal center cells in VΗ/Eμ/VΗ/Δ mice. C, Pie charts, as in B, but of exon sequences for IgG1 cDNA cloned from VΗ/Δ WT spleen cells.
**The Eμ-deficient allele is highly mutated in germinal center B cells of Peyer's patch**

To study the effect of Eμ deletion on SHM, we first analyzed the IgH genes of germinal center B cells isolated from Peyer's patches. B220<sup>CD95<sup>PNAn<sup>high cells were isolated, and PCR was used to amplify *V<sub>μH</sub>*B1–8 coding sequences ("exon" in Fig. 1A) and 214 bp immediately downstream ("intron" in Fig. 1A). The *V<sub>μH</sub>* and *V<sub>μΔ</sub>* alleles are identical throughout the sequenced region (574 bp), but they can be differentially cloned using appropriate primers (no. 4 + no. 3 for *V<sub>μEμ</sub>; no. 4 + no. 2 for *V<sub>μΔ</sub>* allele) (Fig. 1A). The alleles were compared in *V<sub>μEμ</sub>/WT* and *V<sub>μΔ</sub>/WT* animals (Supplemental Table I) and in a more direct comparison in *V<sub>μEμ</sub>/V<sub>μΔ</sub> heterozygous mutant animals (Table I).

As shown in Table I, the *V<sub>μΔ</sub>* and *V<sub>μEμ</sub>* alleles in *V<sub>μΔ</sub>/V<sub>μEμ</sub>* Peyer’s patch cells sustained significant mutation. This finding is consistent with a previous study that demonstrated that SHM takes place at normal frequency on a *V<sub>μ</sub>* knock-in allele (36). However, there was a small, but significant, difference in mutation frequency between the *V<sub>μΔ</sub>* and *V<sub>μEμ</sub>* alleles (p < 0.05; two-tailed t test and Mann–Whitney U test). The mutation frequency on the *V<sub>μΔ</sub>* allele was reduced relative to the *V<sub>μEμ</sub>* allele within the exon (3.73 versus 5.14%) and the intron (2.14 versus 3.28%). A lesser mutation frequency for the *V<sub>μΔ</sub>* allele was also found in the Peyer’s patch cells of *V<sub>μEμ</sub>/WT* and *V<sub>μΔ</sub>/WT* animals, but the difference between the *V<sub>μΔ</sub>* and *V<sub>μEμ</sub>* alleles generally lacked statistical significance (Supplemental Table I).

Germinal center cells were also analyzed in spleens of mice immunized with SRBCs or with the cognate Ag for B1–8V<sub>H</sub> and statistical significance (Supplemental Table I).

**We also examined the effect of Eμ deletion on SHM as revealed in secondary response: IgG1-producing B cells**

We also examined the effect of Eμ deletion on SHM as revealed in a secondary response to Ag. In these experiments, mice were immunized with NP-CGG or with the cognate Ag for B1–8V<sub>H</sub> and λ1 (the hapten NP in NP-CGG) (37, 38). Mutation levels in splenic germinal center B cells were much less than those in the germinal center cells from Peyer’s patches, as was reported previously (17). Under both immunization conditions, the calculated mutation frequency was always lower within the *V<sub>μ</sub>* exon of the *V<sub>μΔ</sub>* allele (for total and synonymous mutations), but the difference between this and the *V<sub>μ</sub>* exon from the *V<sub>μEμ</sub>* allele failed to reach statistical significance (Supplemental Table II).

**Dramatic decrease in SHM on an Eμ-deficient allele in secondary response: IgG1-producing B cells**

We also examined the effect of Eμ deletion on SHM as revealed in a secondary response to Ag. In these experiments, mice were immunized with NP-CGG in alum and boosted one or two times at 3-wk intervals. As in the primary immunizations, NP-CGG was used in these experiments in an attempt to maximally recruit B1–8V<sub>H</sub>-expressing B cells into germinal centers. RNA was isolated from the spleens of immunized mice; a primer specific for the γ1 C region and another specific for V186.2 (the germline *V<sub>H</sub>* gene segment of B1–8V<sub>μ</sub>) were used to amplify V186.2-γ1 transcripts. Because γ1 transcripts derived from the *V<sub>μEμ</sub>* and *V<sub>μΔ</sub>* alleles were indistinguishable, these experiments were carried out independently in *V<sub>μEμ</sub>/WT* and *V<sub>μΔ</sub>/WT* mice rather than in *V<sub>μEμ</sub>/V<sub>μΔ</sub> mice.

As we demonstrated previously, a pronounced difference between *V<sub>μEμ</sub>/WT<sub>b</sub>* and *V<sub>μΔ</sub>/WT<sub>b</sub>* mice is that in B cells from the former mouse strain, exclusion of the WT Ig<sub>H</sub><sup>b</sup> (WT<sub>b</sub>) allele is almost complete, whereas in *V<sub>μΔ</sub>/WT<sub>b</sub>* mice, ~20% of B cells express the modified allele and a fully assembled Ig<sub>H</sub> gene on the WT Ig<sub>H</sub><sup>b</sup> allele (31, 32). We showed that this difference in phenotype arises at the pre-B to immature B cell transition. For the present studies, this failure of allelic exclusion meant that in *V<sub>μΔ</sub>/WT* mice, we could measure SHM on two alleles expressed in the same mouse: one allele with and the other without Eμ.

To compare mutation frequencies for the *V<sub>μΔ</sub>* WT, and *V<sub>μEμ</sub>* alleles in the γ1 transcripts, only the V186.2 gene segment was analyzed (because only this region, and not D and J sequences, was shared among all three alleles). The unique V–D and D–J junction sequences of B1–8V<sub>H</sub> and an engineered mutation in its frame-work region 3 served to unequivocally distinguish transcripts from the *V<sub>μEμ</sub>* and *V<sub>μΔ</sub>* alleles from those deriving from the WT Ig<sub>H</sub><sup>b</sup> allele (31, 32).

As summarized in Table II, 315 mutations were observed among 51 unique cDNA sequences cloned from WT mice, yielding an overall SHM frequency of 2.10%. This frequency is consistent with results from previous studies in WT mice, using a similar strategy (39). In *V<sub>μEμ</sub>/WT* mice, 250 mutations were found among 47 clones derived from the *V<sub>μEμ</sub>* allele, yielding an overall mutation frequency on the *V<sub>μEμ</sub>* allele (1.81%) that was similar to that of the WT Ig<sub>H</sub> allele. Most, but not all, clones isolated from *V<sub>μEμ</sub>/WT* mice were derived from the *V<sub>μEμ</sub>* allele, consistent with the allelic exclusion observed in these animals (31).

When heterozygous *V<sub>μΔ</sub>/WT* mice were analyzed, 93 V186.2-γ1 cDNA sequences derived from the *V<sub>μΔ</sub>* allele contained only 161 mutations (overall mutation frequency: 0.59%), representing a decrease in overall mutation frequency to one third that of the *V<sub>μEμ</sub>* and WT alleles (*p* < 0.001; one-way ANOVA with posttest) (Table II). Because we were also able to isolate cDNA sequences derived from the WT Ig<sub>H</sub><sup>b</sup> allele in the RNA samples prepared from these mice (presumably the products of double-producing, allelically included B cells), it was possible to compare, in the same animals, SHM on the *V<sub>μΔ</sub>* allele with that on the WT allele, where Eμ remained intact. Thirty-nine unique clones carrying *V<sub>μEμ</sub>* sequences and derived from the WT Ig<sub>H</sub><sup>b</sup> allele were isolated. They carried a total of 193 mutations, yielding an overall mutation frequency of 1.68%. This was significantly greater than the mutation frequency of the *V<sub>μEμ</sub>* allele from the same mice (*p* < 0.001). It was comparable to the mutation frequency of the WT alleles in WT mice and to the *V<sub>μEμ</sub>* allele in *V<sub>μEμ</sub>/WT* mice.

### Table I. SHM in germinal center B cells isolated from Peyer’s patches

<table>
<thead>
<tr>
<th>Mouse Genotype</th>
<th>Allele</th>
<th>Region Analyzed</th>
<th>Mutated Clones/Number Clones Sequenced (%)</th>
<th>% Mutation Frequency (No. of Mutations/Total nt Sequenced)</th>
<th>Point Mutations Per Sequence</th>
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<tr>
<td><em>V&lt;sub&gt;μEμ&lt;/sub&gt;/V&lt;sub&gt;μΔ&lt;/sub&gt;</em></td>
<td><em>V&lt;sub&gt;μEμ&lt;/sub&gt;</em></td>
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<td>46/49 (94)</td>
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<td>Synonymous&lt;sup&gt;b&lt;/sup&gt;</td>
<td>44/49 (90)</td>
<td>6.51 (253/3,887)</td>
<td>0–19</td>
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<tr>
<td><em>V&lt;sub&gt;μΔ&lt;/sub&gt;</em></td>
<td><em>V&lt;sub&gt;μEμ&lt;/sub&gt;</em></td>
<td>Intron&lt;sup&gt;c&lt;/sup&gt;</td>
<td>42/49 (86)</td>
<td>3.28 (344/10,486)*</td>
<td>0–24</td>
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<td>Exon&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>3.73 (564/15,120)*</td>
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<td></td>
<td></td>
<td>Synonymous&lt;sup&gt;b&lt;/sup&gt;</td>
<td>29/42 (69)</td>
<td>4.68 (156/3,332)</td>
<td>0–15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Intron&lt;sup&gt;c&lt;/sup&gt;</td>
<td>30/42 (71)</td>
<td>2.14 (192/8,998)*</td>
<td>0–20</td>
</tr>
</tbody>
</table>

<sup>a</sup>360 bp covering B1–8 V<sub>H</sub> coding sequences.

<sup>b</sup>9.33 synonymous sites within the exon [synonymous sites are defined as sites at which nucleotide change(s) will not affect amino acid sequence].

<sup>c</sup>214-bp intronic sequences adjacent to V<sub>H</sub> coding sequences.

<sup>*p* < 0.05. All three sets of sequences (exons, synonymous bases in exons, and introns) were compared between the *V<sub>μΔ</sub>* and *V<sub>μEμ</sub>* alleles (two-tailed Mann–Whitney U test).
Table II.  **SHM in a secondary response (IgG1-producing spleen cells)**

<table>
<thead>
<tr>
<th>Mouse Genotype</th>
<th>Allele</th>
<th>Region Analyzed</th>
<th>Mutated Clones/Number of Clones Sequenced (%)</th>
<th>% Mutation Frequency (Mutations/Total nt Sequenced)</th>
<th>Point Mutations/Sequence</th>
<th>% Mutation Frequency in Mutated Clones a</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>WT</td>
<td>V186.2 b</td>
<td>48/51 (94)</td>
<td>2.10 (315/14,994)</td>
<td>0–30</td>
<td>2.23 (315/14,112)</td>
</tr>
<tr>
<td></td>
<td>V186.2 b</td>
<td></td>
<td>35/47 (74)</td>
<td>1.81 (250/13,818)</td>
<td>0–20</td>
<td>2.43 (250/10,290)</td>
</tr>
<tr>
<td></td>
<td>V186.2 b</td>
<td></td>
<td>26/47 (55)</td>
<td>1.88 (58/3,086)</td>
<td>0–6</td>
<td>3.40 (58/17,077)</td>
</tr>
<tr>
<td></td>
<td>Synonymous b</td>
<td></td>
<td>55/93 (59)</td>
<td>0.59 (161/27,342)**</td>
<td>0–13</td>
<td>1.00 (161/16,170)**</td>
</tr>
<tr>
<td></td>
<td>WT</td>
<td>V186.2 b</td>
<td>38/39 (97)</td>
<td>1.68 (193/11,466)</td>
<td>0–13</td>
<td>1.73 (193/11,172)</td>
</tr>
<tr>
<td></td>
<td>Synonymous b</td>
<td></td>
<td>28/39 (72)</td>
<td>1.91 (49/2,561)</td>
<td>0–3</td>
<td>2.66 (49/1,839)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Mouse Genotype</th>
<th>Allele</th>
<th>Region Analyzed</th>
<th>Mutated Clones/Number of Clones Sequenced (%)</th>
<th>% Mutation Frequency (Mutations/Total nt Sequenced)</th>
<th>Point Mutations/Sequence</th>
<th>% Mutation Frequency in Mutated Clones a</th>
</tr>
</thead>
<tbody>
<tr>
<td>V186.2 b</td>
<td>Synonymous b</td>
<td></td>
<td>25/93 (27)</td>
<td>0.70 (43/6,107)**</td>
<td>0–4</td>
<td>2.62 (43/1,642)</td>
</tr>
<tr>
<td></td>
<td>WT</td>
<td>V186.2 b</td>
<td>38/39 (97)</td>
<td>1.68 (193/11,466)</td>
<td>0–13</td>
<td>1.73 (193/11,172)</td>
</tr>
<tr>
<td></td>
<td>Synonymous b</td>
<td></td>
<td>28/39 (72)</td>
<td>1.91 (49/2,561)</td>
<td>0–3</td>
<td>2.66 (49/1,839)</td>
</tr>
</tbody>
</table>

SHM analysis of cDNA clones. Mice were immunized and boosted with NP-CGG. V186.2-g1 cDNA was cloned from splenic RNA, sequenced, and analyzed for mutation (Materials and Methods).

*Frequency calculated from clones carrying one or more mutations (total number of point mutations/total base pairs sequenced in mutated clones).

*Germline gene sequence for WT analyses; comparable sequences in B1-8 gene for V186.2 and V186.2 knock-in alleles.

*Calculations of mutation frequency included the 65.67 synonymous sites within V186.2 coding sequences [synonymous sites are defined as sites at which nucleotide change(s) will not affect amino acid sequence].

*p < 0.01; ***p < 0.001, compared with WT alleles. (In V186.2/WT cells) and WT alleles (in WT cells and V186.2/WT cells). Kruskal–Wallis test with Dunn’s posttest was performed; no significant differences between WT and V186.2 alleles.

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In Peyer’s patch cells of WT and V186.2 b mice, the reduced mutation frequency on the V186.2 b allele was largely attributable to an increase in clones with no mutation. Although, overall, the V186.2 b allele accumulated smaller numbers of mutations than did the WT or V186.2 b alleles (Fig. 1B, 1C, pie charts), most striking was the larger proportion of clones derived from this allele that had sustained no mutation. Among the exon sequences analyzed in Peyer’s patch cells of V186.2 b mice, 29% (12/42) were without mutation when derived from the V186.2 b allele, whereas only 6% (3/49) lacked mutation when derived from the WT allele (p < 0.05) (Fig. 1B, Table I). Similarly, 29% (12/42) of intron clones derived from the V186.2 b allele showed no mutation, compared with 14% (7/49) from the WT allele (p < 0.1). Because the V186.2 b and V186.2 b sequences were cloned from the same cell population, the difference in the proportion of unmutated sequences from the two alleles could not be explained by differences in cell-sorting purity. This pattern was also seen in the IgG1 transcripts recovered from V186.2 b/WT cells. Although 41% of γ1 transcripts from the V186.2 b allele had no mutations, only 3% of such transcripts derived from the other, WT allele, remained unmutated (Fig. 1C, Table II). Together, these results suggest that the V186.2 b allele is at a competitive disadvantage with respect to recruitment of the SHM machinery.

**Eμ deletion does not affect the distribution or spectrum of mutations**

Two hallmarks of SHM are its preference for RGYW/WRCY sequences (mutation hotspots) and its bias toward transitions (reviewed in Refs. 1–3). Mutations in all alleles analyzed (in Peyer’s patch germinal center B cells and splenic γ1 producers) were preferentially found within RGYW/WRCY sequences (40–50% mutations), and ≥50% were transitional mutations (Supplemental Table III). The base-exchange pattern was also analyzed, and the V186.2 b allele showed the same mutation pattern as the other alleles analyzed (Supplemental Table IV). In summary, the mutations recovered in these analyses were typical of SHM and did not differ in the absence of Eμ.

**μ mRNA level is not reduced in Eμ-deficient B cells**

SHM frequency was shown to positively correlate with transcription level (13). For example, in the Igk locus, deletion of the intronic enhancer (iEκ) had no effect on SHM frequency nor on transcription of assembled Igκ genes, but deletion of 3′Eκ reduced both (17). By analogy, the small decreases in SHM seen upon Eμ deletion could be due to a concomitant decrease in transcription. We previously showed that surface IgM and μ mRNA levels in resting, splenic B cells are as high (or higher) in homozygous V186.2 b mice as in homozygous V186.2 b mice (31). To investigate the possibility that this might differ for activated B cells (undergoing SHM and CSR), we measured μ mRNA levels (by quantitative RT-PCR) in LPS-stimulated B cells and in germinal center cells isolated from Peyer’s patches. As shown in Fig. 2A, μ mRNA levels were as high in V186.2 b/WT B cells as they were in V186.2 b/WT B cells, after LPS stimulation. In germinal center B cells (B220+PNA high CD95+) isolated from Peyer’s patches, V186.2 b/WT

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**FIGURE 2.** IgH gene-expression levels of the V186.2 b and V186.2 b alleles. A. μ-mRNA levels in LPS-stimulated B cells. Data were obtained from three mice of each genotype. B. μ- and α-mRNA levels in Peyer’s patch germinal center cells. μ- and α-mRNA levels in V186.2 b/WT B cells set to 1; data obtained from three pools of four to six V186.2 b/WT mice each and two pools of four to six V186.2 b/WT mice each. p < 0.03. α- and μ-mRNA levels in A and B were measured by quantitative RT-PCR. C. Nuclear run-on assays of IgH transcripts from the V186.2 b and V186.2 b alleles in LPS-stimulated B cells. Results from two experiments (left and right panels) are shown. In each experiment, nuclei were isolated from pools of four animals for each genotype. Filters blotted with DNA of the indicated sequences (actin = positive control; plasmid = negative control).
cells expressed as much µ mRNA and more α mRNA than did $V_{H\mu}/V_{H\alpha}$ germinal center B cells (Fig. 2B).

Nuclear run-on assays were used to more directly measure the rate of transcription of the $V_{H\Delta}$ and $V_{H\mu}$ alleles. Nuclei were isolated from LPS-stimulated, splenic B cells from homozygous $V_{H\Delta}/V_{H\Delta}$ and $V_{H\mu}/V_{H\mu}$ mice, respectively. $IgH$ transcripts that had been initiated before cell lysis were allowed to elongate in the presence of $[^{32}P]UTP$, and the labeled, nascent transcripts were hybridized to an excess of DNA on filters. As shown in Fig. 2C, transcripts complementary to $V_{H}$ and intronic DNAs were as abundant in $V_{H\Delta}/V_{H\Delta}$ nuclei as they were in $V_{H\mu}/V_{H\mu}$ nuclei, demonstrating no Eμ-dependent increase in $IgH$ transcription rate in activated spleen cells (quantification of the signals yielded no statistically significant difference; data not shown).

These data, as well as our previously published studies of resting B cells, demonstrate that transcription of the Iμ gene created by B1–8I $V_{H}$ insertion does not decrease with Eμ deletion at the resting B cell stage nor after B cell activation. Therefore, any decrease in SHM seen upon loss of Eμ cannot be attributed to a decrease in transcription of the affected IgH gene.

**CSR is less efficient in the absence of Eμ**

As an indirect measure of CSR, we analyzed Ig isotype levels in the sera of hemizygous ($V_{H\mu}/\Delta I_{H}$ versus $V_{H\Delta}/\Delta I_{H}$) and homozygous ($V_{H\mu}/V_{H\mu}$ versus $V_{H\Delta}/V_{H\Delta}$) mice. As shown in Fig. 3A, although IgM levels were not affected by Eμ deletion, the levels of almost all other isotypes were slightly lower in mice bearing the $V_{H\Delta}$ allele (as measured by median value), but the difference between mice with the $V_{H\mu}$ and $V_{H\Delta}$ alleles reached statistical significance in only one case (IgG2a, homozygous animals, $p < 0.05$; Kruskal–Wallis test). IgG2b levels were lower than WT in $V_{H\mu}/\Delta I_{H}$ and $V_{H\Delta}/\Delta I_{H}$ hemizygous mice, with the decrease in $V_{H\Delta}/\Delta I_{H}$ mice reaching statistical significance (IgG2b; $p < 0.05$).

In a more direct measure of CSR, splenic B cells homozygous for the $V_{H\mu}$ or the $V_{H\Delta}$ allele were stimulated in vitro with LPS in combination with T cell-derived cytokines that promote IgH class switching (IL-4, IFN-γ, or TGF-β). As shown in Fig. 3B, the percentage of cells that switched from µ to another isotype did not differ significantly between homozygous $V_{H\mu}$ and $V_{H\Delta}$ B cells; however, in every case, there were more switched cells in the homozygous $V_{H\mu}$ B cell cultures.

We took advantage of allelic inclusion in $V_{H\Delta}/WT\alpha$ mice to directly compare CSR on the $V_{H\Delta}$ allele with CSR on the WTb allele. Double producers from these mice ($\mu^{a}\mu^{b}$) were isolated by flow cytometry and then stimulated in vitro to induce CSR. Purity of the sorted $\mu^{a}\mu^{b}$ cells was >98% (e.g., Fig. 4B). Because allelotype-specific Abs are available for IgG2a, $\mu^{a}\mu^{b}$ cells were stimulated to switch to this isotype, and resulting cultures were analyzed to determine whether both alleles switched to IgG2a to the same extent. As shown in Fig. 4A, the anti-allelotype Abs showed good specificity (control cultures consisted of IgG1 cells and IgG2b cells, and both alleles of the IgGa+ cells switched to the IgG2a isotype. The results of four independent experiments (splenches from two $V_{H\Delta}/WT \text{~animals pooled in each experiment}$) showed consistently greater switching on the IgG2a WT allele, but the difference was not statistically significant (Fig. 4A, graph).

To look more broadly at switching on the two alleles, allelespecific loss of surface IgM was used as an indicator of CSR in another series of experiments. We reasoned that if the $V_{H\Delta}$ and WTb alleles switched at the same efficiency, switch-induced $V_{H\Delta}/WT\alpha$ B cell cultures should generate a subpopulation negative for $\mu^{a}$ and $\mu^{b}$ (both alleles having switched to another isotype) and roughly equal subpopulations identified as $\mu^{a}\mu^{b}$ (CSR on WTb allele only) and $\mu^{a}\mu^{b}$ (CSR on $V_{H\Delta}$ allele only), respectively.

However, as shown in Fig. 4B, after stimulation, a $\mu^{a}\mu^{b}$ subpopulation (CSR on WTb allele only) was clearly evident, but no matching $\mu^{a}\mu^{b}$ subpopulation (CSR on $V_{H\Delta}$ allele only) was found. This suggested higher-efficiency switching on the allele that retained Eμ, although it is possible that the $\mu^{a}\mu^{b}$ cells in these cultures derived from contaminating single-producer cells. Arguing against this possibility was the purity of the beginning population (e.g., ~0.7% cells in $\mu^{a}\mu^{b}$ gate, Fig. 4B), as well as the larger proportion of $\mu^{a}\mu^{b}$ cells in the cultures after stimulation (7–10% $\mu^{a}\mu^{b}$ cells; Fig. 4B).

To directly compare the CSR efficiency of two alleles with the same IgH promoter and in a competing environment, we analyzed in vitro-stimulated $V_{H\mu}/V_{H\Delta}$ B cells. In these experiments, CSR was measured by loss of an EcoRI/BamHI fragment that spans Sp4 and, therefore, is lost upon CSR. This fragment is of different sizes on the two alleles (6.2 kb on $V_{H\mu}$ allele, 8.3 kb on $V_{H\Delta}$ allele; Fig. 4C). When $V_{H\mu}/V_{H\Delta}$ B cells are stimulated to switch isotype, the Sp4-region EcoRI/BamHI fragment should diminish in equal measure from the two alleles if both alleles are undergoing CSR at the same frequency. To test this, IgG3+ and IgG3− cells were sorted from LPS-stimulated $V_{H\mu}/V_{H\Delta}$ cultures. DNA isolated from these cell populations was analyzed in Southern blots for the presence of the allele-specific EcoRI/BamHI fragments. As shown in Fig. 4C, both fragments were much more diminished in LPS/IgG3+ cells compared with $V_{H\mu}/V_{H\Delta}$ liver cells (equal amounts of DNA were loaded in each gel lane, and a DNA probe hybridizing to sequences 3′ of the IgH locus served as a DNA loading
control; Supplemental Fig. 1). Therefore, in most IgG3⁺ cells, both alleles had undergone CSR. In contrast, the ratio of the remaining fragments was changed from that of liver. Although the both alleles had undergone CSR. In contrast, the ratio of the fragments from IgG3⁺ cells (upper left panel). The VHΔ fragment/VHΔEµ (and WT fragment/VHΔEµ fragment) ratio was 0.93–0.94 for liver DNA, it was 1.4 and 1.5 for IgG3⁺ cells from VHΔ/VHΔEµ mice (two independent experiments). DNA samples from the IgG3⁺ cells, sorted side-by-side with the analyzed IgG3⁺ cells, as expected, largely retained the same region EcoRI/BamHI fragments from both alleles (Fig. 4C). The VHΔ/VHΔEµ fragment ratios in DNA from these cells were similar to those for liver DNA (0.97/1.00). Therefore, as with SHM, CSR remains robust in the absence of Eµ; however, as also seen with SHM, an Eµ-deficient allele seems to be at a disadvantage when competing with an allele that retains Eµ.

Discussion
A long-standing question regarding the SHM of Ig genes is how this machinery manages to target the IgH, Igk, and Igλ loci, largely leaving other loci untouched (or at least unmodified) (reviewed in Ref. 40). As one of the first tissue-specific, cis-control elements identified in the murine IgH locus, Eµ was an obvious suspect for recruiting the SHM machinery. The Eµ knockout/VHΔEµ knock-in model used in the current study is particularly advantageous for testing that hypothesis. The VHΔ allele carries a functional VHΔ produces IgH chain at levels sufficient to drive B cell development and, therefore, generates normal numbers of peripheral B cells. A matched, Eµ-containing allele (VHΔEµ) makes it possible to compare the behavior of alleles with and without Eµ, where the same DNA sequences (VHΔ and intron) lie at the same distance from the same VHΔ promoter. Moreover, as we have demonstrated, it is often possible to compare these alleles in the same mice and even in the same B cells. This contrasts with prior mouse studies involving Eµ deletion in which B cell development was impaired (in homozygous mutant mice), yielding a small and possibly unusual peripheral B cell compartment, or in which the mutant allele was always nonfunctional (heterozygous mutant mice) (25, 26, 29).

Using the matched-allele system, we found that Eµ deletion results in a small, but significant, reduction in SHM frequency (Fig. 1B, 1C, Tables I, II). The reduced frequency of mutation on the VHΔ allele seems to be largely due to an increase in the proportion of such alleles that escape mutation altogether. The implication is that loss of Eµ results in significantly less efficient recruitment of the SHM machinery. However, once recruited, the pattern of mutation is indistinguishable from WT (Supplemental Tables III, IV). The increase in unmutated clones is not likely an artifact of cloning. Among clones with mutations, duplication was rare: only 1 mutated sequence was isolated twice among 56 unique, mutated sequences recovered from VHΔ/WT cells, suggesting that, similarly, the clones with no mutations were mostly independently derived (data not shown). It is also unlikely that the difference in mutation rates for the VHΔ allele was the result of Ag-driven selection processes that favor one allele over the other because their VHΔ sequences are identical. Moreover, when only synonymous mutations within the V186.2 sequences of WT, VHΔ, and VHΔEµ alleles were considered, the mutation rate remained significantly lower on the VHΔ allele (Table II). The difference in mutation frequency for the VHΔEµ and VHΔ alleles was less pronounced in Peyer's patch B cells than in the usual peripheral B cell compartment, or in which the mutant allele is nonfunctional (heterozygous mutant mice) (25, 26, 29).
deficient mice (29). The investigators attributed this increase (relative to WT mice) to impaired B cell development (and the presence of fewer peripheral B cells), concluding that there was no real change in SHM. However, an alternate interpretation is that the cEμ-deficient allele, like the VqΔ allele of the current study, was less efficiently targeted by the SHM machinery for mutation; however, the already described drawbacks of the earlier experimental system made this difficult to confirm. Findings of a study of SHM in cell culture (hybridoma-expressing human AID) are consistent with the latter interpretation (cEμ deletion resulted in a significant reduction in SHM) (41). However, inconsistent with our findings was the fact that SHM remained at normal levels when cEμ and its flanking MARs were deleted together. The Eμ deletion of the current study also included cEμ and its flanking MARs. However, two of the complicating factors in the cell culture studies were additional modifications made to the IgH locus (in addition to Eμ deletion) and the large variation in IgH gene expression seen among mutant clones (e.g., clones with the same mutation gave rise to IgM-producing and IgM-nonproducing subclones) (41). In summary, previous studies provided hints that Eμ contributes to the efficiency with which the SHM machinery targets an expressed IgH gene; however, the current study, because of the more stringently controlled comparisons possible, gives greater power to that assertion.

Cloned V186.2-γ1 cDNA sequences from the VγEμ allele included more unmaturated sequences than did those from the WT alleles of WT mice (Table II). This might reflect a partial impairment in SHM even for the VγEμ allele (a developmental imprinting problem caused by a shorter stay at the pro-B cell stage as a consequence of Vγ knock-in?); but this interpretation is not supported by the fact that the VγEμ allele was highly mutated (comparable to WT frequencies) in VγEμ/VqΔ Peyer’s patch B cells. Another possibility is that the B cells in WT/WT mice are under stronger selection pressure than are the B cells carrying a knock-in allele; consistent with this interpretation, when only synonymous sites were analyzed, the percentage of unmaturated sequences from the WT and VγEμ alleles did not differ appreciably.

Eμ’s effect on SHM efficiency does not derive from its function as a transcriptional enhancer; transcripts from the VqΔ allele were as abundant as those from the VγEμ allele, and transcription rate was indistinguishable between the two alleles within the sensitivity of a nuclear run-on assay (Fig. 2). This is not a surprising finding, given that previous studies by us and other investigators showed that Eμ is not required to maintain high levels of IgH transcription in Ig-secreting cells (reviewed in Ref. 42). Eμ-independent transcription is explained by activity of the 3′RR, which becomes highly active as early as the immature B cell stage of development (43, 44). It was proposed that the 3′RR is more important than Eμ in driving IgH transcription in the late stages of B cell maturation, and this recently found additional support in the phenotype of 3′RR-deficient mice in which Igμ transcripts were greatly decreased in unstimulated and LPS-stimulated splenocytes (in contrast to no decrease upon Eμ deletion) (24, 31; current study).

Because Eμ deletion does not result in a reduction in transcription rate, we conclude that Eμ’s role in SHM is not secondary to its function as a transcriptional enhancer. This does not mean, of course, that transcription rate cannot affect SHM rate. This correlation has been well-established (e.g., Ref. 17). Recently, deletion of the 3′RR was shown to decrease SHM within IgH genes, and it may well be that its influence on SHM is secondary to its effect on transcription (23, 24).

Given the complexity of the endogenous IgH locus and the relatively small effects seen upon deletion of any individual cis-element, it is reasonable to conclude that there are many layers, and perhaps several redundant layers, to the regulation of SHM targeting (reviewed in Ref. 40). Although replaceable, Vq promoters might provide some specificity to the targeting process because not all strong promoters can support efficient SHM (14). The 3′RR may influence SHM by way of its effect on IgH transcription in mature B cells (24). Finally, the current study suggests that Eμ also contributes to SHM, but in this case it is not through its effect on IgH transcription. Rather, Eμ seems to be operating through a different mechanism, perhaps as a recruitment site/organizing center for attracting one or more components of the SHM machinery. It might be that all of these elements of the IgH locus, and perhaps others yet to be identified, culminate in the locus specificity of the SHM process.

The other aspect of Eμ function addressed in the present matched-allele study was Eμ’s contribution to CSR. The suggestion that Eμ played a role in this process first arose from the analysis of Eμ-deletion chimeras (B cells heterozygous for Eμ deletion in RAG-2 knockout mice) and then the analysis of mice with germline Eμ mutation (25, 26, 29). When made heterozygous with a WT allele, the Eμ-deficient allele was greatly impaired with respect to CSR-like recombination events. This suggested that Eμ was required for CSR, perhaps because of the Iμ promoter that lay within it. By analogy, knockout experiments removing the intronic promoters (Ips) for the germline transcripts associated with other Cμ genes led to selective deficiencies in CSR, affecting only the Cμ with the missing Ip (e.g., Ref. 45). However, unlike the Ips of other Cμ genes, Iμ is within the Iγ locus transcription unit so that transcripts initiating from the promoter of an assembled Vγ gene will, like Iμ transcripts, extend through the Sμ region. Therefore, it might be expected that IgH transcripts that result after V-gene splicing will not supplant the need for Iμ transcripts. Consistent with this hypothesis, there was a severe defect in CSR on alleles that lacked Eμ and an assembled Vγ gene, whereas the current study revealed only a slight defect in CSR when a similar Eμ-deficient allele was supplied with an assembled Vγ gene (and promoter). Interestingly, IgH transcripts through the Sμ region are much more abundant than are Iμ transcripts (35).

However, Eμ deletion in the presence of an assembled Vγ gene is not innocuous with respect to CSR. We consistently observed suboptimal CSR on the VγΔ allele compared with Eμ-sufficient alleles (Figs. 3, 4). Although the mechanism through which Eμ supports optimal CSR is not immediately obvious, one possibility is that removal of the Iμ promoter changes the splicing pattern of μ transcripts and this, in turn, negatively affects CSR. The donor-splice site of the noncoding exon of Iμ transcripts lies close to Sμ (Fig. 1A). This splice site remains on the ΔEμ allele, but it may rarely be used when in the presence of the donor-splice site at the border of a functional Vγ gene. Splicing has long been suggested to play a regulatory role in CSR, and a recent model emerged in which it is suggested that transcription and the subsequent re-arrangement of splicing factors serve to attract AID (35, 46, 47). Perhaps the difference in proximity to Sμ of the two competing donor-splice sites (Iμ exon versus Vγ exon) translates to a difference in AID recruitment and CSR efficiency.

Alternatively, Eμ could affect CSR through its interaction with 3′RR. 3′RR is a primary regulator of CSR and physically interacts with the Eμ region in splenic B cells, as assessed by chromosome conformation-capture assay (48, 49). Although Wuerffel et al. (48) showed that 3′RR continued to interact with an Eμ fragment minus the core (cEμ deletion), it is possible that the larger Eμ deletion of the current study (involving cEμ and flanking MARs) would have some effect on such cross-locus interactions and, thereby, influence the efficiency of CSR.
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Disclosures
The authors have no financial conflicts of interest.

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