A role for the IgH intronic enhancer Eμ in enforcing allelic exclusion

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The intronic enhancer (Eμ) of the *immunoglobulin heavy chain* (*IgH*) locus is critical for V region gene assembly. To determine Eμ's subsequent functions, we created an *Igh* allele with assembled V_H gene but with Eμ removed. In mice homozygous for this Eμ-deficient allele, B cell development was normal and indistinguishable from that of mice with the same V_H knockin and Eμ intact. In mice heterozygous for the Eμ-deficient allele, however, allelic exclusion was severely compromised. Surprisingly, this was not a result of reduced suppression of V-DJ assembly on the second allele. Rather, the striking breakdown in allelic exclusion took place at the pre-B to immature B cell transition. These findings reveal both an important role for Eμ in influencing the fate of newly arising B cells and a second checkpoint for allelic exclusion.

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Abbreviations used: 3′ RR, 3′ regulatory region; BCR, B cell receptor; ES, embryonic stem; IgH, Ig heavy chain; mRNA, messenger RNA.

Ig heavy chain (IgH) genes are assembled through an ordered process of joining V, D, and J gene segments into a functional V region–coding sequence (VDJ recombination). A regulatory element, Eμ, lying just downstream of the J segments, was initially discovered as a transcriptional enhancer (1–3), but Eμ deletion studies subsequently demonstrated that it was essential for promoting efficient VDJ recombination as well (4–8). A model emerged in which Eμ promoted heavy chain variable region (V_H) gene assembly and then served to enhance transcription of the newly formed IgH gene.

Although recombination can theoretically occur on either or both *Igh* alleles, only one allele is expressed in individual B cells, a phenomenon which is termed "allelic exclusion" (9). Allelic exclusion insures that individual B lymphocytes express antigen receptors (Ig) of a single antigen specificity, which is a fundamental requirement of the clonal selection theory put forth by MacFarlane Burnet (10) over 50 yr ago.

Several models have been proposed to explain allelic exclusion. In all models, it is assumed that VDJ recombination occurs at a low frequency and that two-thirds of such rearrangements result in a nontranslatable reading frame (nonfunctional rearrangement). Although these assumptions predict that cells with functional rearrangements on both alleles will be rare, they do not explain their almost complete absence from the normal B lymphocyte repertoire. Models to explain the absence of such cells fall into two general groups: those that invoke selective processes acting at the level of the cell and those that invoke feedback mechanisms affecting VDJ recombination itself. An early model in the former group proposed that expression of μ chains derived from both *Igh* loci would lead to toxic heavy chain levels resulting in cell death (11). Gene targeting experiments in which both *Igh* loci were modified to carry preassembled V_H genes contradicted this model; in almost all B cells of these animals, both *Igh* alleles were expressed (12).

Studies of transformed precursor B cells and of Igμ transgenic mice supported an alternate regulated model of V-DJ recombination (13, 14). In this regulated model, it was proposed that μ chain expression from a productive *Igh* allele prevents V-DJ recombination on the other, precluding development of allelically included B lineage cells (9, 15). In Igμ transgenic mice, DJ assembly is common on the endogenous *Igh* loci of mature B cells, but full VDJ assembly is rare, suggesting that early expression of the Igμ transgene suppresses the second step (V-DJ recombination) in V_H gene assembly within the endogenous loci. Although allelic exclusion is rarely perfect in transgenic mice (16), it has been thought that this results from the variable

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levels and/or timing of transgene expression as compared with IgH genes lying in their natural position within the *Igh* locus. Consistent with the "level" hypothesis, doubling a multicopy Ig transgene by mating led to both higher transgene expression levels and greater allelic exclusion than when the same transgene multimer was left hemizygous (17). Allelic exclusion appeared virtually complete when a preassembled variable region gene was inserted into one allele of the natural *Igh* locus by gene targeting (12).

The μ heavy chain encoded by a newly formed Igμ gene assembles with surrogate light chain (composed of VpreB1/2 and λ5) and with the signaling molecules Igα and Igβ to form a pre-B cell receptor (BCR) (18; for review see reference 19). It is through components of the preBCR that developing B cells sense successful assembly of an Igμ gene on one allele and signal arrest of any further assembly on the other allele (for review see references 19, 20). Even within the context of this feedback regulation, however, there would be opportunity for cells to arise with two functional Igμ alleles, if gene assembly and Igμ-mediated signaling were sufficiently separated in time.

In the present study, we provide evidence that a breach of allelic exclusion occurs quite regularly at the level of V_H gene assembly in developing B cells but, under normal circumstances, this results in few, if any, detectable double producers. When the first allele to assemble a V_H gene lacks Eμ, however, there is a dramatic increase in double producers both within the BM and among peripheral B cells. This striking effect is not the result of a failure to inhibit V_H assembly on the second chromosome; rather, the change takes place at the pre-B to immature B cell transition. One of Eμ's functions, subsequent to VDJ recombination, is to facilitate the survival of newly generated B cells expressing a single *Igh* allele. In its absence, a second checkpoint for allelic exclusion is breached.

RESULTS

Generating a mouse strain that carries an Eμ-deficient V_H-assembled Igh^a allele

Because VDJ recombination is rare on an allele lacking Eμ, B cell development is dramatically impaired in mice homozygous for this deletion (7, 8). This has made it difficult to assess Eμ's functions subsequent to V_H gene assembly, particularly because it is possible that special circumstances (e.g., compensatory mutations) are required to generate the assembled IgH genes found in such animals. To circumvent this problem and also to study the behavior of an Eμ-deficient allele in competition with a WT one, we generated mice bearing an *Igh* allele that lacked Eμ but included a fully assembled V_H upstream of the constant region (C_H) gene cluster. Both core Eμ and flanking matrix attachment regions were deleted, each of which has been shown necessary for the expression of Igμ transgenes in developing B cells (21, 22).

A 3.7-kb region (including the most proximal D_H gene segment, DQ52, all the J_H gene segments, and Eμ) was replaced with a loxP-flanked neomycin resistance gene (neo^R) and a fully assembled V_H gene (neo^RV_HΔ^a; Fig. 1, gene targeting strategy). The V_H gene and ~2 kb of 5′ flanking sequence were isolated from the B1–8 hybridoma, which produces a 4-hydroxy-3-nitrophenyl acetyl-binding antibody (23, 24). Embryonic stem (ES) cells (E14.1 line) were transfected with the targeting vector, and clones undergoing the desired gene replacement were recovered by drug selection.

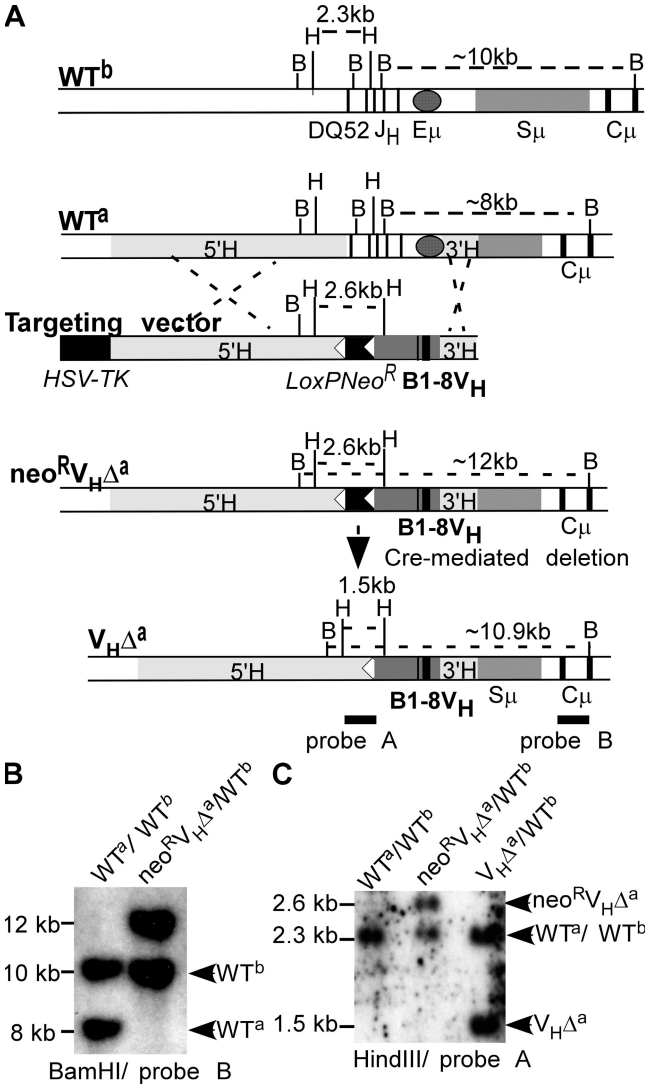

Figure 1. Eμ deletion and V_H gene insertion in an *Igh*^{a} locus. (A) Targeting strategy. WT^b, WT *Igh*^{b} locus. Thick vertical bars show exons for DQ52, J_{H1-4}, and the first two exons of Cμ. Sμ, μ switch region; shaded oval, Eμ; B, BamHI; H, HindIII; WT^a, unmodified *Igh*^{a} locus. Shaded boxes (5′H and 3′H) show regions of homology with targeting vector. Targeting vector: LoxP*Neo*^{R}, loxP-flanked neomycin resistance gene (white arrowheads indicate loxP sites), B1-8V_H promoter region (shaded), and coding sequences (thick vertical lines). neo^RV_HΔ^a: *Igh*^{a} allele after homologous recombination. V_HΔ^a, modified *Igh*^{a} allele after neo^R gene deletion. (B) Southern blot of liver DNA from WT (WT^a/WT^b) and mouse heterozygous for modified *Igh*^{a} allele before neo^R deletion (neo^RV_HΔ^a/WT^b). (C) Southern blot of liver DNA from WT^a/WT^b, neo^R V_HΔ^a/WT^b, and heterozygous mutant mice after neo^R deletion (V_HΔ^a/WT^b).

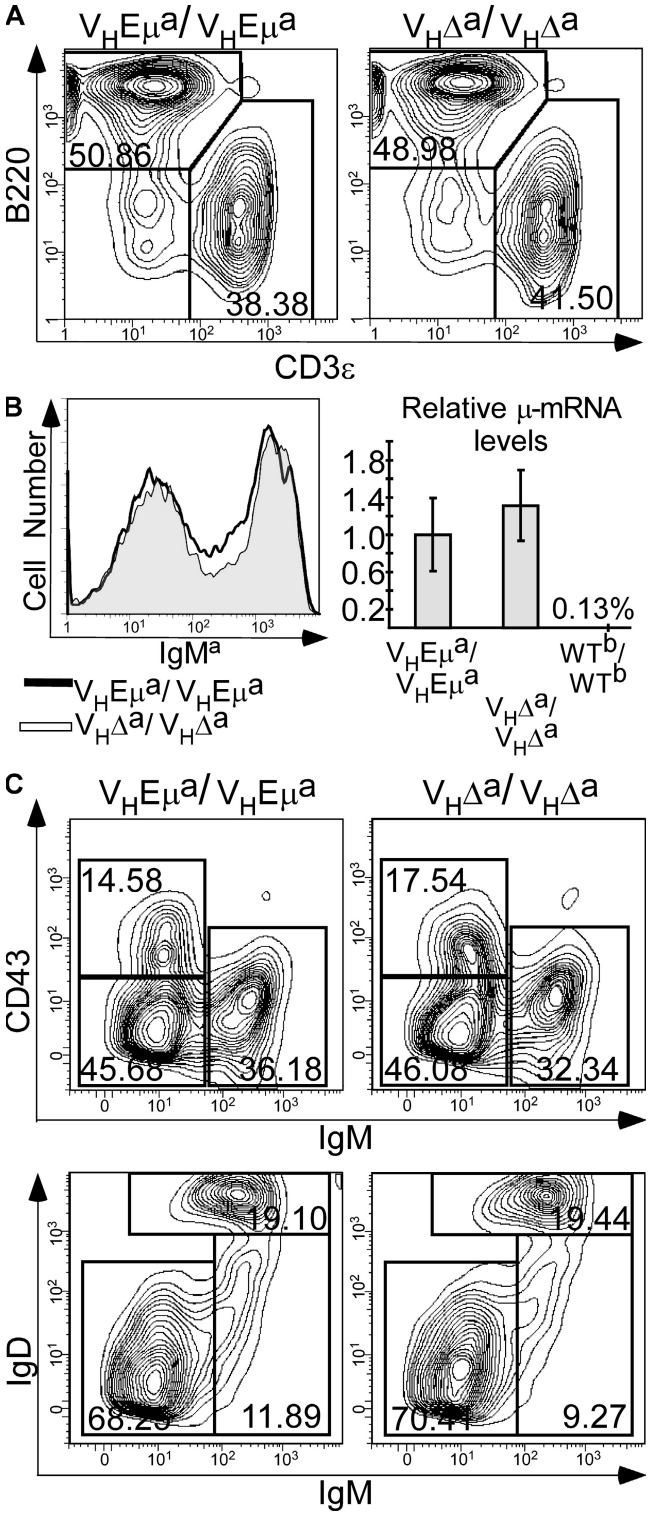

Figure 2. Spleen and BM B cell profiles in homozygous $V_HE\mu^a$ and $V_H\Delta^a$ mice. (A) Flow cytometry profiles of splenic lymphocytes from adult mice stained for B220 (B cell marker) and CD3-ε (T cell marker). (B) Surface IgM^a and relative Igμ transcript levels in splenic B cells. Left, histogram of spleen cells stained with antibody to IgM^a. Right, quantitative RT-PCR results, using primers for B1-8i μ mRNA and normalized to *hgprt1* mRNA (see Materials and methods). μ mRNA from $V_HE\mu^a$ B cells are set as 1.0 and WT^b/WT^b is the negative control. (C) BM B cell subsets. Top, BM cells gated for $B220^+$ cells and analyzed for CD43 and IgM expression. Bottom, BM $B220^+$ cells analyzed for IgD and IgM. Data shown are representative of three animals of each genotype.

Igh locus structure in the ES clones and resulting mouse line was confirmed by genomic Southern blot (Fig. 1, A and B). An 8-kb BamHI fragment spanning Eμ and the first two exons of Cμ in the germline Igh^a locus (WT^a) was replaced with a 12-kb BamHI fragment in the targeted locus of $neo^RV_H\Delta^a/WT^b$ heterozygous mice. The 10-kb BamHI fragment derives from the WT Igh^b allele (WT^b). $neo^RV_H\Delta^a/WT^b$ mice were mated to the EIIa-*cre* transgenic mouse line (25) to remove loxP-flanked neo^R. A DNA probe (probe A) upstream of loxP-flanked neo^R detected a 2.6-kb HindIII fragment from the $neo^RV_H\Delta^a$ locus that was reduced to 1.5 kb upon neo^R deletion ($V_H\Delta^a$ allele; Fig. 1, A and C). neo^R deletion was also confirmed by PCR (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20081202/DC1; and see Materials and methods).

IgM from an Eμ-deficient allele can drive B cell development

It has been assumed that one of Eμ's important roles within the *Igh* locus is to activate transcription of newly assembled IgH genes. Other enhancers that can drive IgH gene transcription lie at the far 3′ end of the locus, but it has not been established whether or not this regulatory region, the 3′ regulatory region (3′ RR), is functional immediately after IgH gene formation in pro-B cells.

If transcription of newly formed IgH genes were Eμ dependent, expression of the $V_H\Delta^a$ allele would be delayed until other transcription control elements (e.g., the 3′ RR) became functional. This would arrest B cell development at the pro-B to pre-B transition in homozygotes but not in mice heterozygous for the $V_H\Delta^a$ allele because, in heterozygotes, VDJ recombination and IgH gene expression could take place on the other WT *Igh* allele. If transcription of newly formed IgH genes were Eμ-independent, B cell development would be normal both in homozygotes and heterozygotes and, because of feedback inhibition of V-DJ recombination, the B cells of $V_H\Delta^a/WT^b$ animals would express only the $V_H\Delta^a$ allele.

B lineage cells were first examined in $V_H\Delta^a/V_H\Delta^a$ animals and directly compared with $V_HE\mu^a/V_HE\mu^a$ animals, a mouse strain which carries the same V_H gene knockin but with Eμ left intact (12). As shown in Fig. 2 A, the proportion of B cells in the splenic lymphocytes of $V_H\Delta^a/V_H\Delta^a$ mice was normal and did not differ from that in $V_HE\mu^a/V_HE\mu^a$ mice (comparisons of multiple mice with these genotypes are summarized in Table S1, available at http://www.jem.org/cgi/content/full/jem.20081202/DC1). IgM^a levels on the surface of resting splenic B lymphocytes was also the same, whether the B cells' *Igh* loci contained or lacked Eμ (Fig. 2 B). Consistent with surface IgM levels, quantitative measurements

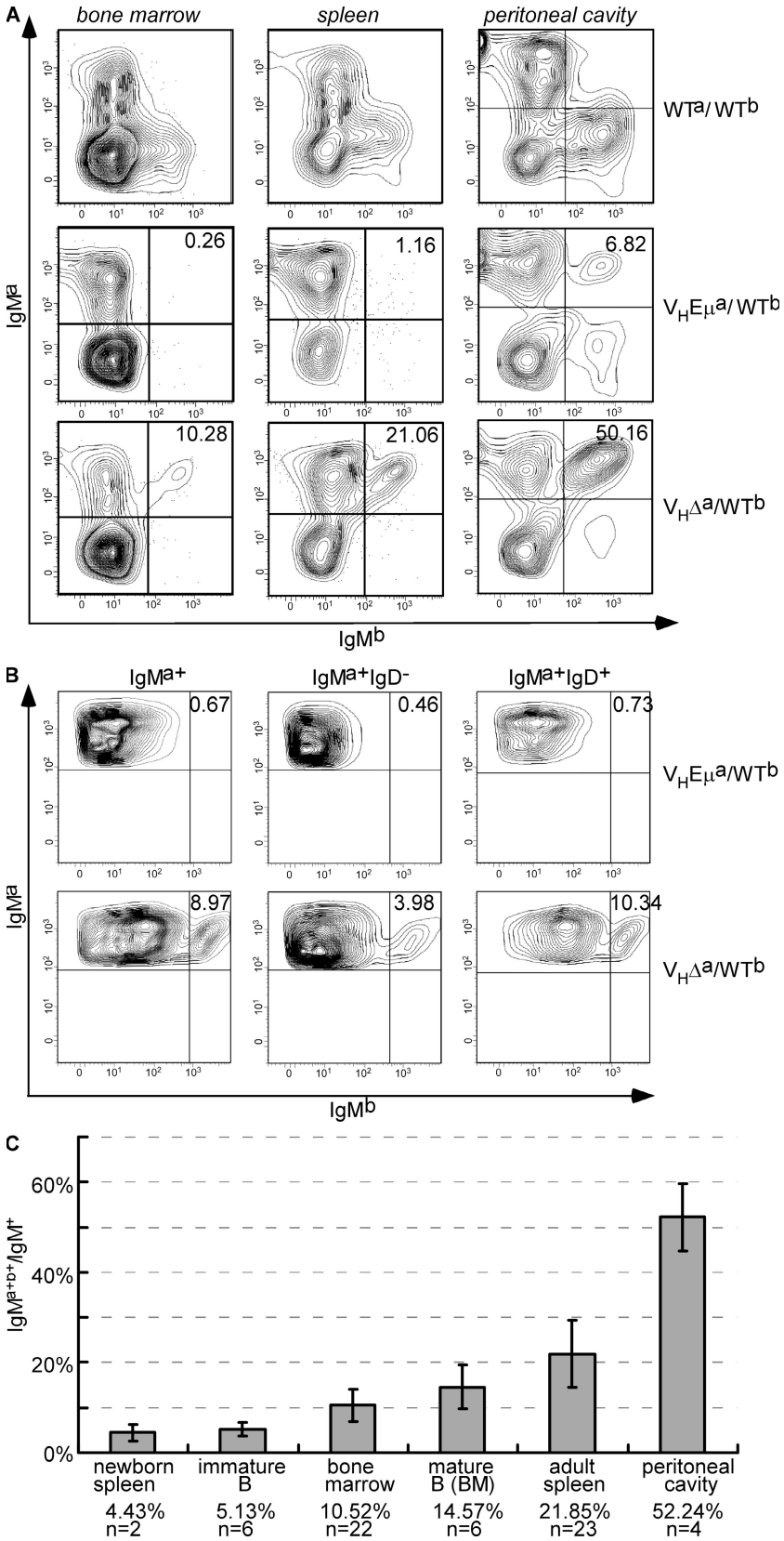

Figure 3. IgM allotype expression in $V_HE\mu^a/WT^b$ and $V_H\Delta^a/WT^b$ mice. (A) Flow cytometry profiles of lymphocytes in BM, spleen, and the peritoneal cavity, stained with antibodies to IgM^a and IgM^b. (B) IgM allotype expression on immature and mature B cells of BM (enriched for $B220^+$ cells). Plots are of

of Igμ messenger RNA (mRNA) produced by splenic B cells showed that the Eμ-deficient genes produced as much (or more) Igμ mRNA as Eμ-containing genes (Fig. 2 B).

To eliminate the possibility that peripheral expansion of a small precursor population was masking a central defect, BM cells were also analyzed in these animals. As shown in Fig. 2 C, the pro-B ($B220^+IgM^-CD43^+$), preB ($B220^+IgM^-CD43^-$), and immature/mature B cell ($B220^+IgM^+CD43^-$) subsets were found in similar proportions in $V_HE\mu^a/V_HE\mu^a$ and $V_H\Delta^a/V_H\Delta^a$ animals (Fig. 2 C, top; summary of data from multiple animals is in Table S1). When antibodies to IgD and IgM were used to distinguish immature (IgM^+IgD^-) from mature B cells (IgM^+IgD^+), these two subpopulations were also found at comparable levels (Fig. 2 C, bottom). The total number of B lineage cells in the BM of these homozygous mutant mouse lines was reduced with respect to WT (≥50% WT; Table S1) but was the same for the two mutant lines, indicating that the difference from WT was a function of the V_H gene knockin by itself and was not affected by the presence/absence of Eμ. We conclude that an assembled IgH gene can be expressed in an Eμ-independent manner early in B cell development, allowing for the normal developmental progression of B lineage cells.

Allelic exclusion is dramatically compromised in the absence of Eμ

It was shown previously that B cells in mice heterozygous for the $V_HE\mu^a$ (knockin) allele expressed only that allele, displaying what appeared to be perfect allelic exclusion (12). Because Igμ from the Eμ-deficient $V_H\Delta^a$ allele could drive normal B cell development in homozygotes, we expected that B cells from mice heterozygous for this locus ($V_H\Delta^a/WT^b$ mice) would similarly express only the $V_H\Delta^a$ allele and not the alternate WT^b allele.

As shown in Fig. 3 A, IgM-positive BM cells from WT^a/WT^b mice expressed either IgM^a or IgM^b but not both. $V_HE\mu^a/WT^b$ BM B cells expressed only the $V_HE\mu^a$ allele, which is consistent with earlier reports (12). Quite clearly, however, BM B cells from $V_H\Delta^a/WT^b$ mice included a subpopulation (~10% of IgM^+ cells) that broke the rules of allelic exclusion, displaying both alleles on the cell surface. This was even more striking in splenic B cells (~20% of IgM^+ cells) and in B cells of the peritoneal cavity (~50% of IgM^+ cells; Fig. 3 A). In stark contrast, $V_HE\mu^a/WT^b$ mice had no distinct population of double producers in the spleen, and such cells, although present in the peritoneal cavity, were much lower in number than in the $V_H\Delta^a/WT^b$ mice (~7% vs. ~50%; Fig. 3 A).

Further analyses of the IgM^{a+b+} peripheral B cells in $V_H\Delta^a/WT^b$ mice revealed that many were of the marginal zone phenotype in spleen and the B1-B cell phenotype in the peritoneal cavity (Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20081202/DC1). In fact, among $CD5^+$ cells in the peritoneal cavity, almost all proved to be double producers. Analyses of fetal liver and newborn spleen, however, provided no evidence that double producers were arising in greater numbers among B1 B cell progenitors (Fig. 3 C and Fig. S3).

To determine the frequency with which double producers were arising in the BM of adult mice, we used IgD/IgM expression to distinguish newly arising immature B cells (IgM^+IgD^-) from recirculating mature B cells (IgM^+IgD^+). Double producers made up a mean of 5% of the immature B cells (Fig. 3, B and C). These expanded to a mean of 15% of mature BM B cells and underwent further expansion in the spleen (Fig. 3, B and C). In summary, analyses of the lymphoid tissues of $V_H\Delta^a/WT^b$ mice revealed a profound defect in allelic exclusion, distinguishing these from $V_HE\mu^a/WT^b$ mice which carried an identical V_H insertion but with Eμ intact.

To confirm at the molecular level that cells staining with both antiallotype reagents in $V_H\Delta^a/WT^b$ animals carried a successfully assembled Igμ gene on the Igh^b allele, splenic IgM^{a+b+} cells were isolated and their genes examined by PCR. A primer specific for the WT *Igh* allele (J_{H4} primer; Fig. 4 D) and primers specific for three V_H families were used to amplify and clone assembled V_H genes on the WT^b allele. 14 independent clones were sequenced, and all revealed a successfully assembled (productive) V_H gene on the WT^b allele (unpublished data).

A productive IgH allele with or without Eμ significantly inhibits, but does not prohibit, DNA rearrangement on the alternate allele

Theoretically, only one-third of V-DJ rearrangements should result in a functional gene. The ~5% double producers among immature BM B cells of $V_H\Delta^a/WT^b$ mice should correspond, therefore, to only 1/3 of the total attempts at V_H gene assembly on the WT^b allele. The vestiges of aberrant rearrangements should be detectable on the WT^b alleles of IgM^{a+b-} single producers in these mice.

To estimate the frequency of such rearrangements, we took advantage of a BamHI restriction fragment length polymorphism within the V-D interval. A probe lying within this interval (Fig. 4 A, probe C) detects a 9.6-kb BamHI fragment in liver DNA from 129/Ola (Igh^a) mice and a 7.5-kb BamHI fragment from C57BL/6 (Igh^b) mice. Because of their location, these BamHI fragments would be deleted upon fusion of any V_H with any D_H gene segment on the Igh^a and Igh^b alleles, respectively.

Splenic B cells were sorted from WT^a/WT^b F_1 animals and from $V_H\Delta^a/WT^b$ mice. IgM^{a-b+} cells from WT^a/WT^b mice almost entirely lacked the BamHI fragment derived

cells gated for IgM^a alone (left), cells that were IgM^{a+} and IgD^- (middle; immature B cells), or IgM^{a+} and IgD^+ cells (right; mature B cells) and then analyzed for IgM^a and IgM^b. In both A and B, the number in top right quadrant = (percentage of double-positive cells)/(total IgM^+ cells). (C) Percentage of IgM^{a+b+}/IgM^+ cells at different stages of development and in different tissues. *n* = number of mice analyzed. Error bars show SD.

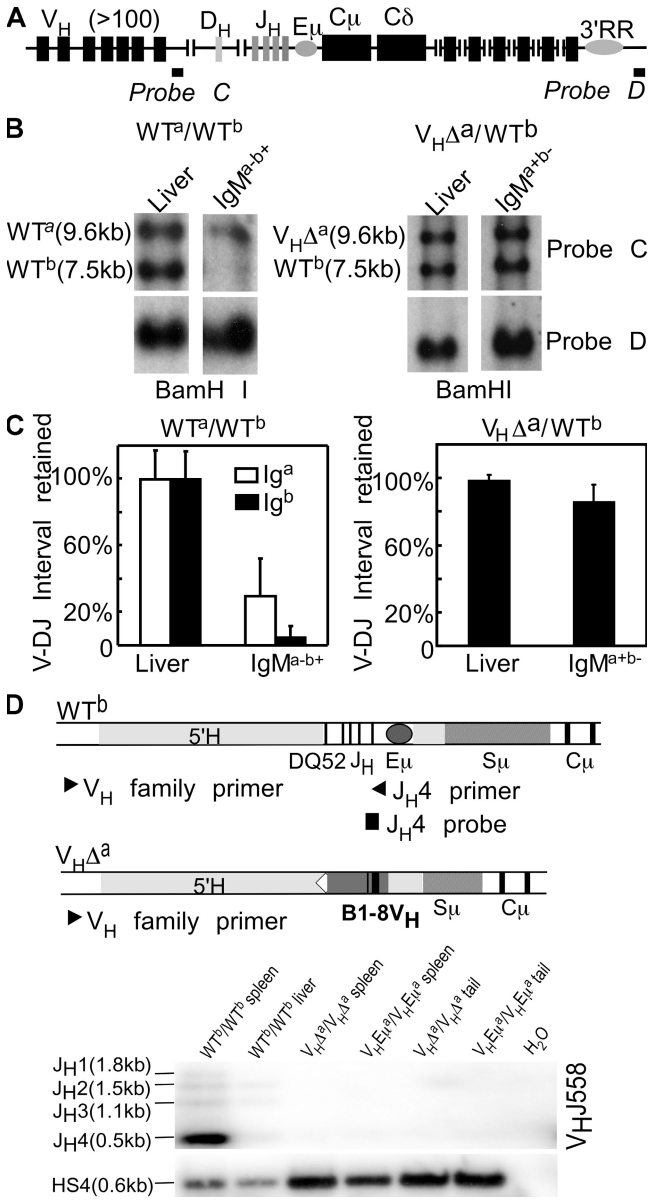

Figure 4. V-DJ rearrangements on the WT *Igh*^{b} allele of $V_H\Delta^a$/WT^b mice. (A) *Igh* locus. Probe C detects a sequence deleted upon V-DJ joining. Probe D detects a sequence retained on both alleles in all cells. (B) Assay for V-DJ rearrangement on the *Igh*^{a} and *Igh*^{b} alleles in WT^a/WT^b and $V_H\Delta^a$/WT^b mice. Genomic DNA extracted from liver and from sorted IgM^{a-b+} spleen cells (WT^a/WT^b; left) and IgM^{a+b-} spleen cells ($V_H\Delta^a$/WT^b; right). DNAs were digested with BamHI and hybridized with probes C (top) and D (bottom). (C) Quantitative analysis (ImageQuant) of blot shown in B. A repeat experiment was done using liver and spleen cells from $V_H\Delta^a$/WT^b animals. Band intensities in liver were set to 100%. Normalization was to probe D for WT^a/WT^b mice; normalization was to the *Igh*^{a} fragment in $V_H\Delta^a$/WT^b mice. Error bars show SD. (D) *Igh* locus maps indicating PCR primers and probes for detecting V-DJ rearrangements on *Igh*^{b} allele. 5′ primers specific for individual V_H gene families (V_H family primer) were used in combination with a 3′ primer (J_H4 primer), which was present on the *Igh*^{b} allele but missing on both the $V_HE\mu^a$ and $V_H\Delta^a$ alleles. The representative blot demonstrates allele-specific amplification. H_2O, no DNA template control.

from the WT^b allele (residual fragment results from contaminating cells in sorted populations) and retained only ~30% germline (liver) levels of the larger BamHI fragment from the WT^a allele (Figs. 4. B and C). A BamHI fragment mapping 3′ of the *Igh* loci (Fig. 4 A, probe D) was used to normalize DNA loading in these experiments. These results are consistent with earlier studies and demonstrate the inefficiency of the V_H assembly process such that many B cells (in this case, ~70%) have undergone unsuccessful VDJ gene assembly on the unexpressed *Igh*^{a} allele before they attempt and succeed at assembly on the *Igh*^{b} allele (13, 26).

In B1–8 V_H knockin mice, the B1–8 V_H gene was inserted upstream of Cμ on the *Igh*^{a} allele and all germline J_H genes were simultaneously removed. As noted earlier, the inserted B1–8 V_H coding sequences were modified to inhibit V_H gene replacement events, so the 9.6-kb BamHI fragment lying between the V_H and D_H genes on this *Igh*^{a} chromosome should not be lost in any B cells from these mice (early studies reported no evidence for V-D rearrangements [reference 13]). As shown in Fig. 4 B, this fragment did, in fact, remain in DNA isolated from IgM^{a+b-} B cells from $V_H\Delta^a$/WT^b mice. The smaller BamHI fragment from the WT^b allele was also present in these cells and was present at 85–95% germline levels (Fig. 4 C). Quantification of the data showed no statistically significant difference from levels of this fragment in liver DNA (Fig. 4 C). We conclude that most cells expressing only the $V_H\Delta^a$ allele have not undergone V-DJ rearrangement on the WT^b allele.

To address this issue further, we turned to a more sensitive measure of V-DJ rearrangement. Semiquantitative PCR was used to quantify VDJ rearrangements on the WT^b allele of IgM^{a+b-} splenic B cells (single producers) using V_H family primers and the *WT Igh*-specific primer described earlier. As shown in Fig. 5 A, V-DJ rearrangements on the WT^b allele were readily detectable in IgM^{a+b+} double producers from the $V_H\Delta^a$/WT^b mice (similar in abundance to *Igh* rearrangements in the IgM^{a+b-} cells of WT mice). PCR products were also generated with each of the V_H family primers when DNA was isolated from the single producers (IgM^{a+b-} B cells) from $V_H\Delta^a$/WT^b spleen, but these were somewhere between 1/5 and 1/25 as abundant as those seen in double producers or in the IgM^{a+b-} B cells from WT mice. Importantly, this small but detectable level of V-DJ rearrangement on the WT^b allele was also seen in the IgM^{a+b-} spleen cells of $V_HE\mu^a$/WT^b mice (Fig. 5 B). Consistent with the surface phenotype, these rearrangements, cloned from both animals, were nonproductive (18/18 from $V_HE\mu^a$/WT^b mice and 18/19 from $V_H\Delta^a$/WT^b mice; unpublished data).

The same PCR approach was used to estimate the frequency of WT^b rearrangements in pre-B cells of both $V_H\Delta^a$/WT^b and $V_HE\mu^a$/WT^b mice. Pre-B cells comprise the pool that has just ceased IgH rearrangement and is beginning the process of Ig light chain gene assembly. Semiquantitative PCR assays of DNA from these cells showed that this pool had undergone D-J joining on the WT^b allele at the same frequency as pre-B cells from normal WT^a/WT^b mice (Fig. 5 C). V-DJ

joins were also evident on the WT^b alleles of pre-B cells from both $V_H\Delta^a/WT^b$ and $V_HE\mu^a/WT^b$ mice. These, however, were both present at ~1/5–1/25 the frequency seen in normal pre-B cells (Fig. 5 D).

To increase the resolution of these analyses, we used two-fold dilutions of template, confirmed linearity (Fig. S4, available at http://www.jem.org/cgi/content/full/jem.20081202/DC1), and performed triplicate PCRs of diluted template samples. Pre-B cells from seven mice of the $V_HE\mu^a/WT^b$ genotype and seven of the $V_H\Delta^a/WT^b$ genotype were examined in three independent pools. Assembly of the WT^b allele in these pre-B cells was compared with that of the two WT (WT^a and WT^b) alleles in WT mice (two pools of two mice each). Representative data are shown in Fig. 5 E and a quantitative analysis of all data is provided in Fig. 5 F.

V-region gene assembly on the WT^b allele in the pre-B cells of both $V_HE\mu^a/WT^b$ and $V_H\Delta^a/WT^b$ mice was reduced relative to the WT^a and WT^b alleles in pre-B cells of WT mice (Fig. 5 F). The reduction, relative to WT mice, was greater for the more distal V_HJ558 family than for the more proximal V_H7183 family. We conclude that the $V_H\Delta^a$ and $V_HE\mu^a$ alleles were inhibiting, but not entirely prohibiting, V-DJ recombination on the allelic chromosome, and the feedback inhibition was largely, if not entirely, Eμ independent. Most importantly, however, the data showed that there was no significant difference in the amount of V-region assembly taking place on the WT^b alleles of $V_HE\mu^a/WT^b$ and $V_H\Delta^a/WT^b$ pre-B cells. Assuming that some of these rearrangements were productive (confirmed by cloning as described in the next paragraph), both populations of pre-B cells included precursors to double-producing cells.

An Eμ-dependent checkpoint for allelic exclusion at the pre-B to immature B cell transition

Because the phenotypic outcome of gene assembly on the WT^b allele was so dramatically different in the $V_H\Delta^a/WT^b$ and $V_HE\mu^a/WT^b$ mice (double producers evident in the immature and mature B cells of the former but not the latter mouse strain), we asked whether the V_H gene rearrangements in the pre-B cells of these two mouse lines differed in some fundamental way. Pre-B cells were harvested from mice of both genotypes, their DNA was isolated, and assembled V_H genes from the WT^b allele were cloned and sequenced. 15/36 unique V_H7183-DJ rearrangements cloned from pre-B cells of the $V_HE\mu^a/WT^b$ genotype were both in frame and lacked a stop codon (productive rearrangements), as were 12/36 unique V_H7183-DJ rearrangements cloned from $V_H\Delta^a/WT^b$ pre-B cells. Closer examination of these productive rearrangements showed no fundamental differences among them in the D_H segments used, the 7183 V_H family members used, or the overall size of the junctions (Table S2, available at http://www.jem.org/cgi/content/full/jem.20081202/DC1). N nucleotides were found in the V-D junctions in >70% of the sequences, and 40% of these had three or more N nucleotides at this junction (mean length, 3.5 nt in $V_HE\mu^a/WT^b$ mice and 4.3 nt in $V_H\Delta^a/WT^b$ mice). Notably, this contrasts with light chain genes where N nucleotides are much more rare (~10% of cells) and are found in much lower numbers/junction (generally 1 or 2 nt/junction) (27). We conclude that the precursors to double producers can be found in BM from both $V_H\Delta^a/WT^b$ and $V_HE\mu^a/WT^b$ mice and that V_H assembly on the WT^b allele has likely occurred before the pre-B cell stage (see Discussion).

In an attempt to understand the mechanism through which Eμ might influence the development and selection of precursor B cells, we compared levels of μ mRNA generated from the $V_HE\mu^a$ and $V_H\Delta^a$ alleles in pre-B cells. To enrich for pre-B cells, $V_HE\mu^a$ and $V_H\Delta^a$ mice were backcrossed to $Rag1^{-/-}$ mice to generate $V_HE\mu^a/WT^b$ and $V_H\Delta^a/WT^b$ mice that lacked Rag-1 activity. In both of the resulting strains, B cell development was arrested at the pre-B cell stage because Ig light chain gene assembly was blocked. Age-matched mice of each genotype were killed and $B220^+$ BM cells isolated (see Materials and methods). As expected, no IgM^+ cells were present in these BM cells, and the bulk of cells were smaller and expressed lower levels of CD43 than comparable cells from $WT^{b/}WT^b$ $Rag1^{-/-}$ littermates, which is consistent with their having progressed to the pre-B cell stage (Fig. 6 A). Total RNA was isolated from the $B220^+$ BM cells of Rag1-deficient $V_HE\mu^a/WT^b$ and $V_H\Delta^a/WT^b$ mice (WT^b/WT^b; $Rag1^{-/-}$ littermates were controls) and Igμ mRNA was quantified by real-time RT-PCR (5′ primer for unique VDJ junction of V_HB1-8 and 3′ primer for C_H1 exon of Cμ; see Materials and methods).

Steady-state μ mRNA levels were roughly twice as high in cells expressing the $V_HE\mu^a$ allele than in cells expressing the $V_H\Delta^a$ allele (Fig. 6 B). This was in contrast to the finding in splenic B cells where the Eμ-deficient allele produced as much or more Igμ mRNA than its Eμ-containing counterpart (Fig. 2 C). Protein levels mirrored the mRNA levels; pre-B cells expressing only the $V_H\Delta^a$ allele produced ~1/2 the amount of Igμ protein produced by cells expressing the $V_HE\mu^a$ allele IgH (Fig. 6 C). At this developmental stage, when pre-BCR and BCR-mediated signals are dictating B cell fate, μ heavy chain levels were measurably influenced by the presence or absence of Eμ.

Evidence for greater selective pressure on the developing B cells of $V_H\Delta^a/WT^b$ mice

The reduced Igμ in $V_H\Delta^a/WT^b$ pre-B cells might be suboptimal for preBCR-mediated clonal expansion and/or BCR-mediated selection into the immature B cell pool. Survival of precursors expressing both alleles (double producers) in $V_H\Delta^a/WT^b$ mice might reflect a need for the increased Igμ levels achieved by biallelic expression. If this were true, IgM^{a+b-} cells might circumvent this problem through alternate means, perhaps by expressing light chains that yielded BCRs with superior signaling properties. This would likely require light chain receptor editing. Receptor editing can be achieved through successive Vκ-Jκ rearrangements in which Vκ-Jκ joins involving the more upstream Jκ gene segments (Jκ1 and Jκ2) are replaced by joins to the more downstream Jκ

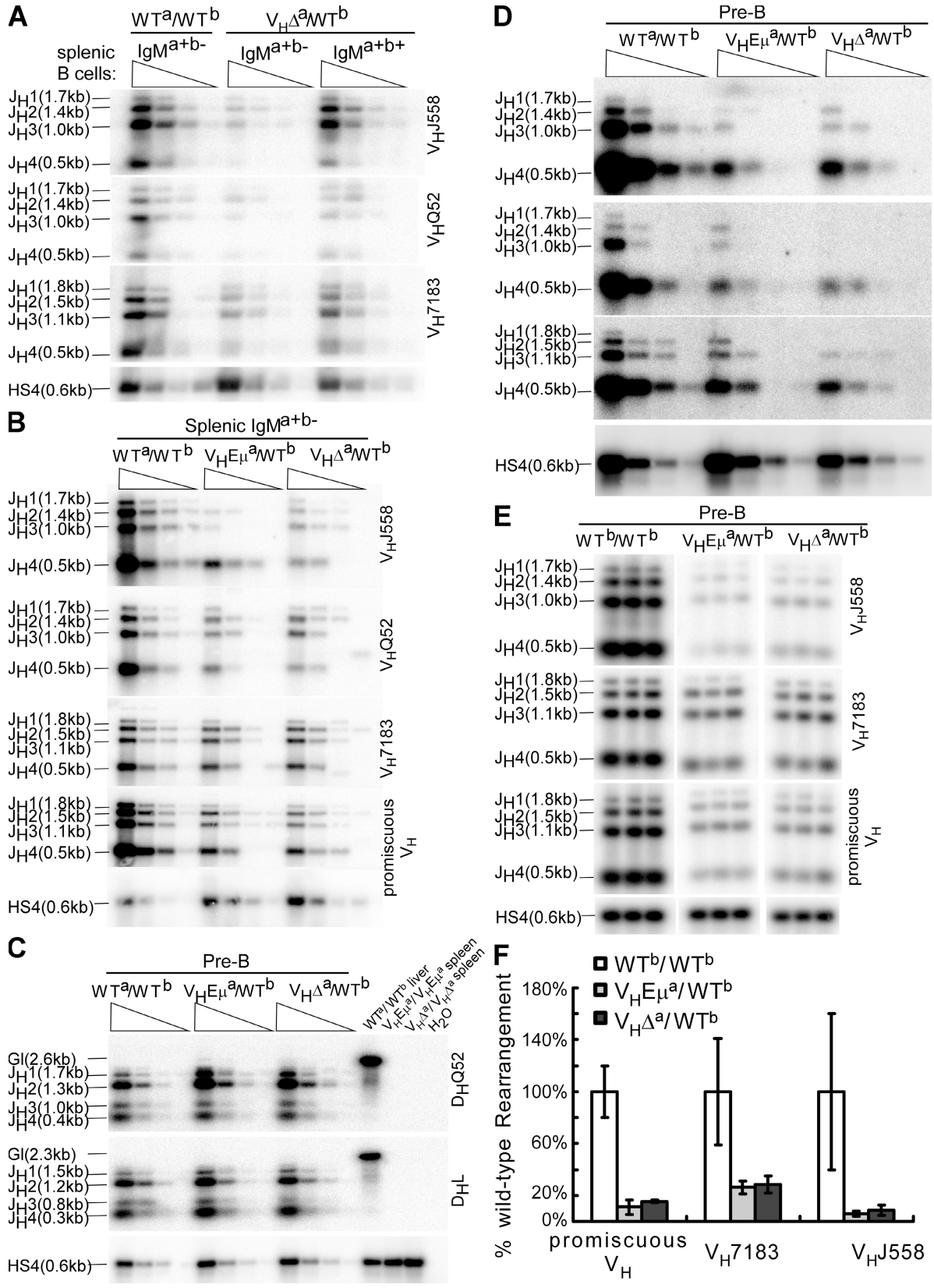

Figure 5. V–DJ rearrangements on the WT *Igh*^{b} allele of $V_HE\mu^a/WT^b$ and $V_H\Delta^a/WT^b$ mice. (A) Splenic B cells from WT^a/WT^b and $V_H\Delta^a/WT^b$, sorted on the basis of phenotype (IgM^{a+b-} or IgM^{a+b+}). PCRs were performed on fivefold serial dilutions of DNA templates (four lanes/cell type). PCR

segments (Jκ4 and Jκ5). A degenerate Vκ primer (VκD) (28) was used in combination with a primer downstream of Jκ5 (Jκ5e) to examine Jκ gene usage in splenic B cells of $V_HE\mu^a$/WT^b and $V_H\Delta^a$/WT^b animals. As shown in Fig. 7 (A and B; and Fig. S5, available at http://www.jem.org/cgi/content/full/jem.20081202/DC1), the IgM^{a+b-} single producers of $V_H\Delta^a$/WT^b mice uniquely showed a decrease in Vκ-Jκ joins involving Jκ1 and Jκ2 gene segments and a concomitant increase in joins involving Jκ5. The IgM^{a+b+} double producers of $V_H\Delta^a$/WT^b mice were not similarly affected; these cells arose from precursors that would have been expressing both the $V_H\Delta^a$ allele and the WT^b allele where Eμ remains intact.

Another indicator of light chain receptor editing is an increase in the numbers of $Ig\lambda^+$ cells. In normal mice, $Ig\lambda^+$ cells make up only ∼5% of splenic B cells, and analyses of Igκ loci in Igλ-producing cells have suggested that *Igλ* genes are usually assembled only after the opportunity to express one of the two available *Igκ* alleles has been exhausted (29; for review see references 30, 31). We looked for Igλ-expressing splenic B cells in WT^a/WT^b, $V_H\Delta^a/WT^b$, $V_HE\mu^a/WT^b$, $V_H\Delta^a/V_H\Delta^a$, and $V_HE\mu^a/V_HE\mu^a$ mice. We also examined hemizygous $V_H\Delta^a/\Delta J_H$ mice (the ΔJ_H allele lacks all J_H gene segments, prohibiting V_H assembly on this allele) (32). The hypothesis was that both the $V_H\Delta^a/WT^b$ and $V_H\Delta^a/\Delta J_H$ mice would have increased numbers of Igλ-expressing B cells because most of the B cells of the former and all B cells of the latter would have arisen from precursors expressing only the $V_H\Delta^a$ allele. Consistent with this hypothesis, the frequency of Igλ producers among $V_H\Delta^a/WT^b$ splenic B cells was significantly higher than that in $V_HE\mu^a/WT^b$ mice (mean of 16 vs. 10%; $P = 0.01$; Igλ expressers were higher in both knockin strains relative to WT, presumably because of constraints imposed by predominant expression of a single structure heavy chain; Fig. 7 C). It was the single producers of the $V_H\Delta^a/WT^b$ mice, and not the double producers, that explained the increase in Igλ–expressing B cells relative to $V_HE\mu^a/WT^b$ mice (Fig. 7 D). Finally, consistent with the stated hypothesis, no such difference was observed when mice homozygous for the knockin alleles were compared (Fig. 7 C). In $V_H\Delta^a/V_H\Delta^a$ mice, biallelic expression of the $V_H\Delta^a$ allele would be expected to equal expression of one allele with Eμ.

DISCUSSION

The present study has shown that the intronic enhancer Eμ serves a critical function even after V_H gene (VDJ) assembly is complete. In mice heterozygous for an Eμ-deficient but productively rearranged IgH gene ($V_H\Delta^a/WT^b$), large numbers of IgH double-producing cells arose in peripheral tissues, breaking the rules of allelic exclusion. In contrast, a matched mouse line with the same V_H knockin but with Eμ present ($V_HE\mu^a/WT^b$) lacked these double-producing cells. In both animals, there was evidence that the Igμ product of the assembled IgH gene was signaling a shutdown of V_H assembly on the Igh^b allele, which is the primary mechanism postulated to ensure IgH allelic exclusion (Figs. 4 and 5). There was no measurable difference in the efficiency of this feedback inhibition whether Eμ was present or absent.

This latter finding was unexpected. Eμ was initially discovered as a transcriptional enhancer and believed to serve as such in newly formed IgH genes (1–3). In that capacity, Eμ could dictate preBCR levels and, thereby, signaling strength in a developing B lymphocyte. Even in Eμ's absence, however, pre-B cells were dominated by those that had undergone D-J, but not V-DJ, rearrangement on their Igh^b alleles, demonstrating that feedback signals inhibiting this last step in recombination were no less efficient in the absence of Eμ.

Both $V_H\Delta^a/WT^b$ and $V_HE\mu^a/WT^b$ mice contained rare pre-B cells with V-DJ rearrangement on their Igh^b alleles. Sequence analyses of these rearrangements revealed abundant N nucleotides in the V-D junction (Table S2), suggesting that they occurred in pro-B cells, the only developmental stage at which terminal transferase (TdT) is expressed (27, 33). Chromatin remodeling studies have documented that pro-B cells are the only developing B cells with contracted *Igh* alleles facilitating V-DJ rearrangements, especially those involving distal V_H gene segments (e.g., V_HJ558) (34). V_H J558-DJ rearrangements on the Igh^b allele were easily detected in pre-B cells of both $V_H\Delta^a/WT^b$ and $V_HE\mu^a/WT^b$ mice. These results suggest that subsequent to V_H gene assembly, there is sufficient delay in the assembly of, or signaling through, the preBCR to allow RAG-mediated rearrangements on the second allele in a minority of cells before transition to the pre-B cell stage.

Signaling through the preBCR is required not only to down-regulate the RAG genes (terminating V-DJ recombination) but also to promote cell proliferation and differentiation

strategy detects V-DJ rearrangements on only the Igh^b allele in $V_H\Delta^a/WT^b$ mice (both alleles in WT^a/WT^b mice). VDJ rearrangements involving each J_H gene segment are indicated (J_H1, J_H2, etc.). V_H family primers are indicated to the right of blots (V_HJ558, etc.). PCR of HS4 (a 3′ RR element) was normalized for DNA input. (B) Like A, but a primer designed to anneal to all V_H families (promiscuous VH 46) was included. (C) D-J rearrangements on the Igh^b allele of pre-B cells ($B220^+CD43^-IgM^-$) sorted from six mice of each genotype. The D_HL primer anneals to most D_H genes (47); DQ52 anneals only to D_HQ52. Allele-specific 3′ primer (J_H4) was used as in A. Liver DNA from WT^a/WT^b mice and spleen cell DNA from homozygous $V_HE\mu^a$ and $V_H\Delta^a$ mice were included as controls. H_2O, no template control. (D) Pre-B cells were isolated as in C and PCR reactions using the V_H gene family primers were performed as described in A. (E) Pre-B cells were isolated as in C. Sample dilutions determined to be in the linear range (Fig. S4, available at http://www.jem.org/cgi/content/full/jem.20081202/DC1) were prepared in triplicate for PCR. Representative data is shown. (F) Quantitative analysis of VDJ rearrangement blots. For each PCR reaction with a given V_H primer, signals for J_H1, J_H2, J_H3, and J_H4 rearrangements were quantified and summed by ImageQuant. Values obtained for pre-B cells from $V_HE\mu^a/WT^b$ and $V_H\Delta^a/WT^b$ mice were normalized to the corresponding values for pre-B cells of WT^b/WT^b mice. Data were obtained from two pools of two mice each for WT^b/WT^b and three pools involving a total of seven mice for each of the $V_HE\mu^a/WT^b$ and $V_H\Delta^a/WT^b$ mouse lines. Error bars show SD.

to the pre-B cell stage (for review see reference 31). If the reduced Igμ chain level in $V_H\Delta^a/WT^b$ pro-B cells resulted in preBCR signals that were suboptimal for promoting this transition, we might expect that precursors to double producers (expressing Igμ from two alleles, one with Eμ present) would be enriched in the pre-B cell population of $V_H\Delta^a/WT^b$ mice. Similarly, if expression of the $V_H\Delta^a$ were delayed relative to expression of the $V_HE\mu^a$ allele, a higher proportion of pre-B cells in $V_H\Delta^a/WT^b$ mice would carry a functionally assembled V_H gene on the WT^b allele, as this would be required in the absence of $V_H\Delta^a$ allele expression for transit to the pre-B cell stage. This was not the case, however; these precursors were present at the same or close to equal numbers in $V_H\Delta^a/WT^b$ and $V_HE\mu^a/WT^b$ mice (Fig. 5 and Fig. S4). Quantitative comparisons of V-DJ assembly on the WT^b allele showed no significant difference between $V_H\Delta^a/WT^b$ and $V_HE\mu^a/WT^b$ pre-B cells, and cloned V_H gene sequences from the WT^b allele had the same ratio of productive to nonproductive rearrangements in both pre-B cell populations. In RAG-$1^{-/-}$ mice, the bulk of B-lineage cells in both mice assumed a pre-B cell phenotype, further supporting the idea that signaling through the Igμ chain (preBCR) to promote the pro- to pre-B cell transition was largely unaffected by loss of Eμ and lower Igμ levels (Fig. 6).

The transition from pre-B to immature B cell requires successful assembly of a functional Ig light chain gene, resulting in replacement of the preBCR with a BCR. The BCR on immature B cells serves two opposing purposes. On the one hand, it is required for survival (BCR ablation at any B cell stage tested leads to cell death), and it has been suggested

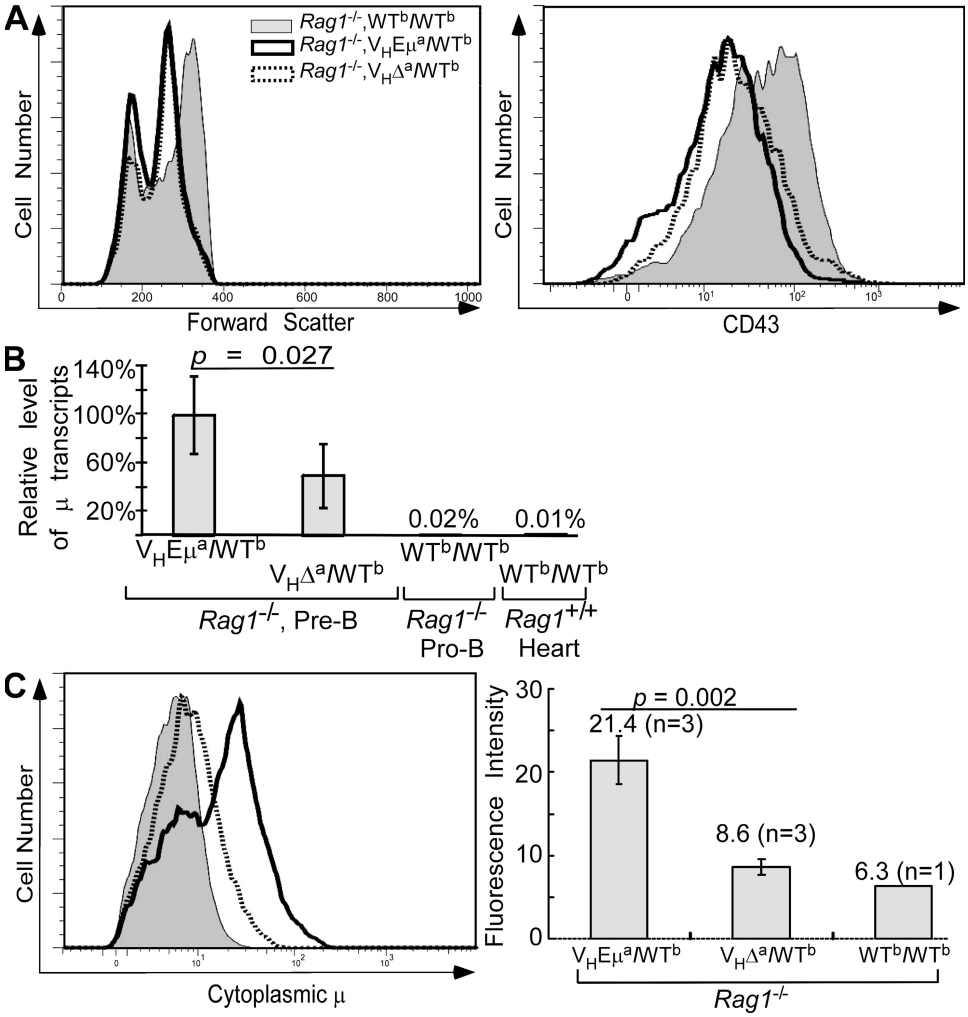

Figure 6. Igμ transcription and cytoplasmic Igμ protein levels in pre-B cells of mutant mice. (A) B220+ lymphocytes from BM of Rag1-deficient WT^b/WT^b, $V_HE\mu^a/WT^b$, and $V_H\Delta^a/WT^b$ mice were analyzed for size (forward scatter) and for CD43 expression by FACS. Data shown are representative of three individual mice of each genotype analyzed. (B) Igμ mRNA levels in cells shown in A. Data were generated by quantitative RT-PCR, normalized to *hgprt1* mRNA, and included two experiments, analyzing a total of five individual animals of each genotype. Negative controls were mRNA isolated from pro-B cells of a WT^b/WT^b *Rag1*$^{-/-}$ littermate and mRNA from C57BL/6 heart tissue (WT^b/WT^b, *Rag1*$^{+/+}$). Statistical significance (P = 0.027) was obtained by a two-tailed Student's *t* test. Error bars show SD. (C) Cytoplasmic Igμ levels in B220+ BM cells of Rag1$^{-/-}$ mice. Left, histograms of cytoplasmic Igμ. Right, mean Igμ fluorescence in pre-B cells from multiple mice (*n* = number of mice analyzed). Error bars show SD.

that this BCR-transmitted tonic signaling is antigen independent (positive selection) (35). On the other hand, if the BCR signal is too strong (e.g., autoreactive BCR), the cell is induced to modify the antigen specificity of the BCR through receptor editing and, failing that, is induced to die (negative selection) (for review see references 30, 31). The nature and strength of the BCR signal in immature B cells, therefore, profoundly affects cell fate. We propose that reduced Igμ levels in emerging immature BM B cells of $V_H\Delta^a/WT^b$ mice result in BCR signaling that is generally below the threshold for positive selection. In these animals, the rare cells that express both *Igh* alleles (double producers) would have a selective advantage because the increased tonic signaling through the BCR could then reach the required threshold for survival.

If, in the absence of Eμ, BCR density is generally not optimal for effective tonic signaling, then how do the single producers in $V_H\Delta^a/WT^b$ animals reach the immature B cell stage? Both density and signaling strength can be affected by light chain through its effects on BCR stability (the strength of heavy/light chain association) and Ag specificity. B cells starting with a disability (low μ-chain levels) would have more stringent light chain requirements than normal, needing either light chains that form a stronger and more stable association with the V_HB1-8-μ chain or those that form an innocuous antigen specificity with stronger basal signaling properties. Analyses of these cells were consistent with that prediction. The IgM^{a+b-} cells in $V_H\Delta^a/WT^b$ and $V_H\Delta^a/\Delta J_H$ mice showed greater use of downstream Jκ gene segments (evidence of successive V-J rearrangements on a single Igκ allele) and included a larger subpopulation of Igλ-producing cells as compared with IgM^{a+b-} cells from $V_HE\mu^a/WT^b$ mice (Fig. 7). Also consistent with this model was the finding that light chain editing was increased neither in the IgM^{a+b+} cells from $V_H\Delta^a/WT^b$ animals (μ-chain emanates from both a WT allele and the $V_H\Delta^a$ allele) nor in the B cells of $V_H\Delta^a/V_H\Delta^a$ mice (μ-chain emanates from two copies of the $V_H\Delta^a$ allele).

As described in the previous paragraph, we predicted that inferior BCR signals caused by reduced Igμ in IgM^{a+b-} cells from both $V_H\Delta^a/WT^b$ and $V_H\Delta^a/\Delta J_H$ mice would lead to light chain editing. Unable to sense that a light chain gene has been successfully assembled, the cell would continue to accumulate V-J rearrangements. A correlation between underexpressed BCRs and light chain editing has been described previously (36, 37). Reciprocally, strong autoreactive BCR signals can also lead to light chain editing. In this case, the cell uses light chain editing to replace the light chain component of an undesirable BCR.

A corollary to this model is that in $V_HE\mu^a/WT^b$ mice, neither single producers nor double producers have a below-threshold signaling problem. Rather, in these mice, threshold signaling is achieved whether expression is monoallelic

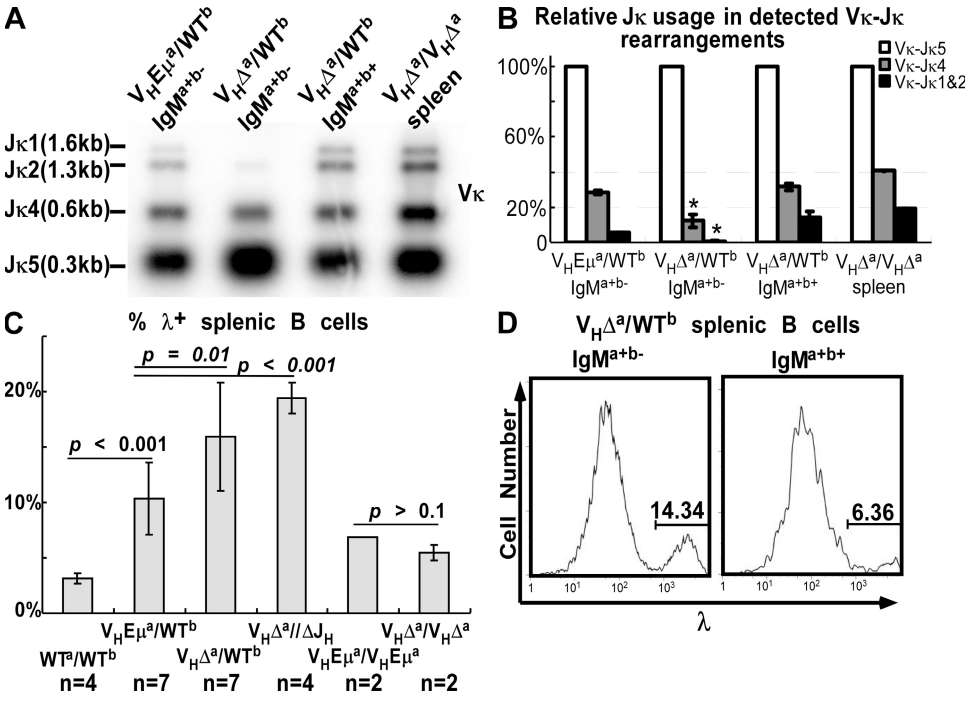

Figure 7. Light chain editing in IgM^{a+b-} cells of $V_H\Delta^a/WT^b$ mice. (A) Vκ-Jκ rearrangement analyses. PCR products detected with Jκ5 probe (see Materials and methods). (B) Quantitative analysis (ImageQuant) of blot shown in A and a repeat experiment. Detected Vκ-Jκ4 and Vκ-Jκ1 and 2 products were normalized to those for Vκ-Jκ5. *, $P < 0.05$ by a two-tailed Student's *t* test. Pie charts in Fig. S3 (available at http://www.jem.org/cgi/content/full/jem.20081202/DC1) show the relative increase in Vκ-Jκ5 rearrangements in IgM^{a+b-} B cells from $V_H\Delta^a/WT^b$ animals. Error bars show SD. (C) λ+ splenic B cells in WT and mutant mice. Spleen cells were stained for both Igκ and Igλ and the percentage of λ+ cells was calculated as λ/(κ+λ). Genotypes are provided below bars. Data were pooled from four experiments. *n* = number of mice analyzed. P-values were calculated by a two-tailed Student's *t* test. Error bars show SD. (D) Histogram of cells stained for μ^a, μ^b, and λ. Left, single producers (IgM^{a+b-}) from $V_H\Delta^a/WT^b$ mice; right, double producers (IgM^{a+b+}) from $V_H\Delta^a/WT^b$ mice.

(single producers) or biallelic (double producers). Cells producing two different heavy chains at equally high levels, however, would be at greater risk of creating a receptor with unacceptable autoreactive properties, placing double-producers in $V_HE\mu^a/WT^b$ mice at a selective disadvantage (i.e., subject to clonal deletion) relative to single producers. It has been estimated that ~75% of newly arising immature B cells harbor an autoreactive BCR (38–40). A cell producing two different heavy chains, each combining with a single unique light chain would have, on average, only a 6% chance (as compared with the usual 25%) of avoiding expression of an autoreactive BCR. Moreover, autoantibody silencing through formation of an alternate light chain (light chain editing) would be compromised in such a cell because a new light chain might solve the problem with one of the heavy chains and yet create a new autoreactive receptor with the other.

In summary, feedback inhibition of V-DJ rearrangement on the WT^b allele is evident in the pre-B cells of both $V_H\Delta^a/WT^b$ and $V_HE\mu^a/WT^b$ mice. Despite this inhibition, $V_H\Delta^a/WT^b$ and $V_HE\mu^a/WT^b$ mice have equal or close to equal numbers of pre-B cells that harbor two functionally assembled *Igh* alleles. Potential antigen specificity for both single and double producers is exactly the same in both animals after assembly with randomly generated Ig light chains. Nevertheless, the newly arising B cells differ significantly between these two animals, with allelic exclusion compromised only in the $V_H\Delta^a/WT^b$ mice. The ultimate effect on the mature B cell pool is profound. As described and shown in Fig. 3 (and Fig. S2), the ~5% double producers among immature B cells in $V_H\Delta^a/WT^b$ mice expanded to comprise ~20% of splenic B cells and ~50% of B cells in the peritoneal cavity. We suggest that the difference in outcome in the $V_H\Delta^a/WT^b$ and $V_HE\mu^a/WT^b$ mice (the immature B cells arising/surviving from the precursors) is a result of selection pressures, both positive and negative, that favor double producers only in $V_H\Delta^a/WT^b$ mice. Allelic exclusion with respect to the *Igh* locus, therefore, can be viewed as, at minimum, a two-step process involving both a largely Eμ-independent (feedback inhibition of V-DJ recombination) and an Eμ-dependent (pre-B to immature B selection) phase. More generally, these results suggest that one of Eμ's important functions is to ensure that monoallelic expression of most newly assembled IgH genes is sufficient to support both B cell development and entry into the mature B cell pool. Anything that reduces that expression (variants in promoter sequences, enhancer sequences, or signaling components) puts the system at risk for promoting the development of allelically included cells with potentially autoreactive properties.

Unlike cells expressing two different light chains in which one is generally expressed on the surface to the exclusion of the other (41, 42), the mature B cells expressing two different heavy chains in $V_H\Delta^a/WT^b$ mice can be expected to express both types of receptor on the cell surface at comparable levels. It is likely of significance to issues of autoimmunity that expression of the $V_H\Delta^a$ allele is low at the time of selection but high once cells reach the mature B cell compartment. Notably, many of the IgM^{a+b+} double producers in these animals occupy the marginal zone of spleen and constitute most, if not all, $CD5^+$ B1 B cells in the peritoneal cavity. It is not clear at this point how precursors of IgM^{a+b+} double producers are selected into these mature B cell compartments. One possibility is that expressing the WT^b allele fulfills the antigen-specificity requirements associated with B1 B cell development (43). As $CD5^+$ B1cells have been shown to require unusually strong and often autoreactive BCR signals for survival (43), it is also reasonable to speculate that monoallelic expression of the $V_H\Delta^a$ allele, even with the assistance of light chain editing, is insufficient to signal development and/or maintenance of this B cell subset, leading to selective expansion of the double producers into this B cell pool.

MATERIALS AND METHODS

Generating $V_H\Delta$ mice

To generate the B1-8VDJΔEμ targeting vector, K. Rajewsky (Harvard Medical School, Boston, MA) and W. Muller (University of Manchester, Manchester, England, UK) provided us with a vector (B1-8iVDJ) (12) that we modified to replace the 3′ homology arm with a 1.4-kb sequence between Eμ and Sμ (nt 3880–5295, GenBank accession no. J00440). Both the B1-8VDJΔEμ and B1-8iVDJ targeting vectors included ~2-kb natural 5′ flanking DNA to the B1-8V_H gene, and the 5′ homology arm was comprised of 9 kb of DNA lying immediately upstream of D_HQ52 in the murine *Igh* locus. B1-8V_H coding sequences carried a TGT to TGC silent mutation at codon 92 to prevent V_H replacement events (12). The ClaI–NotI fragment, serving as 3′homology arm in the B1-8iVDJΔEμ targeting vector, was generated by PCR, using DNA from the 129P2/OlaHsd-derived ES (E14.1) cell line as template. Primers used were the following: 5′-CTCATCGATTCGGTTGAACATGCTGGTTG-3′ (ClaI site underlined) and 5′-CTCGCGGCCGCAGTGTAGGCAGTAGAGTTTA-3′ (NotI site underlined).

The B1-8VDJΔEμ targeting vector was used to transfect ES cell line E14.1, and three appropriately targeted clones were used to generate chimeric mice (Gene Targeting and Transgenic Service Laboratories, Rockefeller University). Successful germline transmission was achieved from one of the lines. Positive offspring that were heterozygous for the $neo^R V_H\Delta$ allele were mated to EIIa-cre mice (C57BL/6J background; provided by H. Westphal, National Institutes of Health, Bethesda, MD) (25) to induce deletion of the neomycin resistance gene. The resulting mouse line was designated $V_H\Delta$.

$V_HE\mu$ mice were previously described as B1-8i mice (12) (supplied by K. Rajewsky, CBR Institute for Biomedical Research, Boston, MA). Mice carrying *Igh* alleles that lacked the J_H gene segments were obtained from The Jackson Laboratory (B6.129P2-Igh-J^{tm1Cgn}/J; stock #002438). The mutant allele lacking the J_H gene segments is called ΔJ_H in the present studies.

The V_H knockin for $V_HE\mu$ was also accomplished in ES 14.1 cells. Both $V_HE\mu$ and $V_H\Delta$ mice were mated to C57BL/6J mice for allelic exclusion analyses. Progeny of intercrosses between $V_HE\mu^a/WT^b$ and $V_H\Delta^a/WT^b$ mice were also analyzed, and double producers were strictly correlated with the $V_H\Delta$ allele. Mice were bred and maintained in animal facilities at Hunter College, City University of New York, and all mouse experiments were approved by the Hunter College Institutional Animal Care and Use Committee.

Southern blots

Genomic Southern blot analyses were performed as previously described (44). ~20 μg of restriction enzyme–digested genomic DNAs were size fractionated on 0.8% agarose gels and the DNA was transferred to nylon membrane (GE Healthcare). Blots were hybridized with ^{32}P-labeled probes generated by the random priming method (MegaPrime; GE Healthcare). Probe A (Fig. 1) is a 1.2-kb XhoI–HindIII fragment cloned from the B1-8iVDJ targeting vector (nt 25622029–25623228; GenBank accession no. NT_166318). Probe B (Fig. 1)

is a 724-bp sequence that begins upstream of Cμ and covers C_H1 and ~1/2 of C_H2 (nt 1214283–1215005; GenBank accession no. NT_114985). Probe B was generated by PCR, using E14.1 ES cell DNA as template. Primers used were the following: CμU1, 5′-CAAGGAAATAGCAGG GTGTAG-3′; and CμD1, 5′-CTTTGTTCTCGATGGTCACC-3′. Probe C (V-D interval; Fig. 4) is a 1,056-bp sequence ~73 kb upstream of the most C_H-distal D gene fragment DFL16.1 and ~25 kb downstream of the most C_H-proximal V gene fragment V_H81X (nt 7471–8526; GenBank accession no. AC073553). Probe C was generated by PCR from C57BL/6 mouse genomic DNA using the following primers: E1f, 5′-CATCCAGATACAGCACTCCCTTGTGTC-3′; and E1r, 5′-GAAGGCCAGGACCAAGGATTGAATAC-3′. Probe D (Fig. 4) is a 1,469-bp sequence derived from a region ~70 kb 3′ of the hs4 element within the *Igh* 3′ RR (nt 25343884–25345352; GenBank accession no. NT_166318). Probe D was generated by PCR from C57BL/6J mouse genomic DNA with the following primers: Df, 5′-CTGAAGTTGGATGTAGGCCT-GAAACTG-3′; and Dr, 5′-CCTCCCAATGCTAAGTAGAAACAGACG-3′. The JH4 probe is a 156-bp probe for J_H4 (nt 134365–134520; GenBank accession no. AC073553) that is used to detect DJ and VDJ rearrangements on the Igh^b allele. The J_H4 probe was generated by PCR from C57BL/6 mouse germline DNA with the following primers: JH4f, 5′-CTATGGACTACTGGGGT-CAAGGAAC-3′; and JH4r, 5′-CAACTTCTCTCAGCCGGCTC-3′. The hs4 probe is a 601-bp probe for the hs4 element of the *Igh* 3′ RR (nt 25418984–25419584; GenBank accession no. NT_166318).

Flow cytometry

Generally, 10^6 cells were incubated at 4°C for 15 min in staining buffer (PBS, 5.6 mM glucose, 0.1% BSA, and 0.1% NaN_3) with monoclonal antibodies (BD or SouthernBiotech). Single-cell suspensions prepared from BM and spleen were treated with ACK buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM EDTA, pH 7.3) to lyse erythrocytes. Cells from the peritoneal cavity were recovered by flushing the cavity with cold RPMI-1640 media (Mediatech, Inc.), containing 5% bovine calf serum (Thermo Fisher Scientific). In most cases, propidium iodide was added before analyses to exclude dead cells.

Side and forward scatter were used to gate on lymphocytes. FACS instruments were used for flow cytometry and sorting (FACScan, FACSCalibur, and FACSVantage; BD). In some cases, BM B lineage cells were first enriched by positive selection (MACS B220 MicroBeads kit; Miltenyi Biotec). Data were acquired with CellQuest or Pro CellQuest (FACS instruments) and then further analyzed with FlowJo software (Tree Star, Inc.).

Monoclonal antibodies were obtained from BD, except where indicated, and conjugated to fluorescein-isothiocyanate (anti–mouse CD3-ε, anti-IgM, anti–mouse IgM^b, anti-IgD, anti–mouse CD21, and anti–mouse Igκ), phycoerythrin (anti–mouse IgM^a, anti–mouse CD23, anti–mouse CD43, and anti–mouse Igλ [SouthernBiotech]), allophycocyanin (anti–mouse B220 and anti–mouse CD5), or biotin (anti–mouse IgM^b, anti–mouse IgM^a, and anti–mouse IgD [Southern Biotech]). Cells were then washed in washing buffer (PBS, 5.6 mM glucose, and 0.1% NaN_3) and biotin-conjugated monoclonal antibodies were revealed with streptavidin-allophycocyanin (BD) or streptavidin-phycoerythrin (BD).

Cytoplasmic Igμ staining

BM cells were incubated with antibodies to surface antigens, washed, and then fixed and stained with rat anti–mouse IgM (eBioscience) using Cytofix/Cytoperm (BD).

PCR

Genotyping mice. All genotyping PCR reactions were performed for 40 cycles with an annealing temperature of 60°C. PCR kits with HotStar Taq polymerase were used (QIAGEN), and the manufacturer's protocol was adhered to. Primers used were the following: number 2, 5′-CAGAGGGAGTTCA-CACAGAGCATG-3′ (within the 3′ homology region of B1-8iVDJΔEμ; nt 136031–136054; GenBank accession no. AC073553); number 4, 5′-TCTT-TACAGTTACTGAGCACACAGGAC-3′ (immediately 5′ of the leader exon of B1-8V_H; nt. 312629–312655; GenBank accession no. BN000872); and number 8 (Eμ), 5′-CTTCCCTCTGATTATTGGTCTCCATTC-3′ (nt 135733–135759; GenBank accession no. AC073553). These three primers were used together. Numbers 2 and 4 generate an 848-bp product from the targeted Igh^a locus, and numbers 2 and 8 generate a 322-bp product from the WT Igh^b locus. The PCR products generated by these three primers (numbers 4, 8, and 2) overlap the region of VDJ insertion.

Confirming neo^R deletion. Primers used were the following: number 9, 5′-CCCACCATCACAGACCTTTCTCCATAG-3′ (within the 5′ homology region of the targeting vectors; nt 132108–132134; GenBank accession no. AC073553); and number 10, 5′-CTGAGGGCAGCAGTA-CAATGATGAGTC-3′ (within the 5′ flank of B1-8V_H and ~280 bp 3′ of loxP-flanked neo^R; primer differs by two nucleotides [underlined] from nt 311488–311514; GenBank BN000872). Primers number 9 and 10 can generate a product only from the targeted Igh^a allele. The PCR product from the $neo^RV_H\Delta^a$ allele was ~1,500 bp before neo^R deletion and, from the $V_H\Delta^a$ allele after neo^R deletion, was 400 bp.

Detecting D-J and V-DJ rearrangements in B-lineage cells. To analyze V-DJ rearrangements in isolated B-lineage cells, a previously described protocol (7) was followed with slight modification. Genomic DNA template was isolated from sorted cells, either by conventional procedures or by the following method: cells were lysed in 10 mM Tris-HCl, pH 8.0, and 0.1 mM EDTA (50 μl/10^5 cells), proteinase K was added to a concentration of 0.5 mg/ml, and the mixture was incubated for 2.5 h at 50 and 95°C for 10 min. Serial dilutions of the purified DNA (or cell lysates) were used as a template to specifically amplify D-J and V-DJ rearrangements derived only from WT *Igh* alleles. Allele specificity was achieved by using a 3′ primer that derived from sequences 3′ of J_H4 and that were missing on the targeted Igh^a allele of both $V_HE\mu$ and $V_H\Delta$ mice. The 5′ primers were "degenerate" primers designed to anneal to the D_H genes, one of three V_H gene families (J558, Q52, or 7183 family), or all V_H genes. Primers for D_H families were the following: The primer for D_HL, 5′-GGAATTCGMTTTTTGTSAAGGGATC-TACTACTGTG-3′, is a degenerate primer that anneals to most murine D_H sequences (45); and the primer for D_HQ52, 5′-CCACAGGCTCGAGA-ACTTTAGCG-3′, anneals to the most J_H-proximal murine D_H sequence, DQ52 (7). Primers for V_H families were the following: V_HJ558 family primer, 5′-GCGAAGCTTARGCCTGGGRCTTCAGTGAAG-3′ (7); V_HQ52 family primer, 5′-GCCAAGCTTCTCACAGAGCCTGTCCATCAC-3′ (7); V_H7183 family primer, 5′-GCGAAGCTTGTGGAGTCTGGGGGAG-GCTTA-3′ (7); and promiscuous V_H primer, 5′-GGGAATTCGAGGTG-CAGCTGCAGGAGTCTGG-3′ (46). Each of these primers was used in conjunction with the following primer, which lies 3′ of J_H4 and cannot anneal to the targeted Igh^a alleles: 5′-AGGCTCTGAGATCCCTAGACAG-3′ (nt 134530–134551; GenBank accession no. AC073553). PCR products were size fractionated on 1.5% agarose gels, blotted, and probed with a ^{32}P-labeled J_H4 probe (described in Southern blots). DNA amounts were normalized to a PCR product derived from the *Igh* locus 3′ RR element hs4 present as one copy/haploid genome in all cells (as in reference 7). HS4 primers used were the following: 5′-CCAAAAATGGCCAGGCCTAGG-3′ (nt 25419564–25419584; GenBank accession no. NT_166318); and HS4-3′, 5′-AGGTC-TACACAGGGGCTCTG-3′ (nt 25418984–25419003; GenBank accession no. NT_166318). PCR conditions for detecting V-DJ and D-J rearrangements were 30 cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 3 min. The exceptions were for data shown in Fig. 5 (E and F) and Fig. S2. In these instances, HS4 was amplified for 20 cycles instead of 30.

Vκ-Jκ rearrangements. The analysis of Vκ-Jκ rearrangements was the same as for IgH VDJ rearrangements except that different primers and probes were used. A degenerate Vκ primer (VκD) (28) was used to detect all Vκ genes, and a primer downstream of Jκ5 (Jκ5e) was used to detect rearrangements, regardless of Jκ fragment used. Primers used were the following: VκD, 5′-GGCTGCAGSTTCAGTGGCAGTGGRTCWGGRAC-3′ (a degenerate primer that anneals to most Vκ gene sequences) (28); Jκ5e, 5′-CTGACACTGTATGCCACGTCAACTG-3′ (downstream of Jκ5; nt 3168393–3168418; Gene ID: 243469; derived from GenBank NC_000072).

The Jκ5 probe used for detecting PCR products was generated by PCR, using the following primer pair: Jκ5f, 5′-GCTCACGTTCG-GTGCTGGGAC-3′ (nt 3168251–3168271; Gene ID: 243469; derived from GenBank NC_000072); and Jκ5r, 5′-ATAATGAGCCCTCTC-CATTTTCTCAAG-3′ (nt 3168367–3168393; Gene ID: 243469; derived from GenBank NC_000072).

Real-time RT-PCR

For analyses of Igμ transcripts in splenic B cells, B cells were enriched by negative selection (B cell isolation kit; Miltenyi Biotech). For analyses of Igμ transcripts in pre-B cells, enrichment was by positive selection for $B220^+$ cells (B220 MicroBeads kit; Miltenyi Biotech). Total RNA was isolated with the RNeasy mini-prep kit (QIAGEN). Real-time RT-PCR analyses of Igμ mRNA transcribed from the $V_H\Delta$ and $V_HE\mu^a$ alleles were performed with the QuantiTect SYBR Green RT-PCR kit (QIAGEN). Igμ transcripts were amplified with a 5′ primer that annealed to the unique VDJ junction sequence of V_HB1-8 and a 3′ primer that annealed to a sequence within the C_H1 exon of Cμ. The VDJ junction primer used was 5′-CGCAA-GATACGATTACTACGG-3′ and the Cμ primer used was 5′-GAAGA-CATTTGGGAAGGACTG-3′ (nt 140198–140218; GenBank accession no. AC073553). Samples were normalized using primers and probes for mRNA from the housekeeping gene *hgprt1* (hypoxanthine/guanine phosphoribosyl transferase; Applied Biosystems; TaqMan gene expression assay ID: Mm03024075_m1) and the TaqMan one-step RT-PCR kit (Applied Biosystems). PCR reactions were performed on the 7500 Real-Time PCR System (Applied Biosystems) and analyzed with a program supplied by Applied Biosystems (User bulletin #2; http://docs.appliedbiosystems.com/pebiodocs/04303859.pdf).

Online supplemental material.

Additional results show PCR-cloning results demonstrating that surface Ig phenotype correlated with cloned IgH genes from sorted spleen cells. Fig. S1 shows evidence of CRE-mediated neo^R deletion. Fig. S2 shows that double producers were enriched among marginal zone B cells in spleen and B1 B cells in peritoneal cavity. Fig. S3 shows analyses of newborn spleen and fetal liver cells for double producers. Fig. S4 shows establishment of quantification in V_H gene assembly PCR experiments. Fig. S5 shows pie charts quantifying relative use of Jκ segments in Vκ-Jκ rearrangements in WT versus mutant mice. Table S1 shows absolute numbers of B cell subsets in BM and B and T cells of spleen in mutant and WT mice. Table S2 shows DNA sequences of assembled V_H genes cloned from WT and mutant mice. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20081202/DC1.

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