Elucidation of IgH intronic enhancer functions via germ-line deletion

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Studies of chimeric mice demonstrated that the core Ig heavy chain (IgH) intronic enhancer (iE) functions in V(D)J and class switch recombination at the IgH locus. To more fully evaluate the role of this element in these and other processes, we generated mice homozygous for germ-line mutations in which the core sequences of iE (ceE) were either deleted (ceEΔ2Δ mice) or replaced with a pgk-Neo cassette (ceEmN mice). The ceEΔ2Δ mice had reduced B cell numbers, in association with impaired D to JH and VD to DJH rearrangement, whereas ceEmN mice had a complete block in IgH V(D)J recombination, confirming that additional cis elements cooperate with iE to enforce D to JH recombination. In addition, developing ceEΔ2Δ and ceEmN B lineage cells had correspondingly decreased levels of germ-line transcripts from the JH region of the IgH locus (μ0 and μ transcripts); although both had normal levels of germ-line Vκ transcripts, suggesting that ceE may influence IgH locus V(D)J recombination by influencing accessibility of μ proximal regions of the locus. Consistent with chimeric studies, peripheral ceEΔ2Δ B cells had normal surface Ig and relatively normal class switch recombination. However, ceEΔ2Δ B cells also had relatively normal somatic hypermutation of their IgH variable region genes, showing unexpectedly that the ceE is not required for this process. The availability of mice with the iE mutation in their germ line will facilitate future studies to elucidate the roles of iE in V(D)J recombination in the context of IgH chromatin structure and germ-line transcription.

germ-line transcription | immunoglobulin heavy chain | somatic hypermutation | VDJ recombination | class switch recombination

The mouse Ig heavy chain (IgH) locus can be divided into two principal regions: an ~2.2-Mb 5’ region containing clusters of variable (VH), diversity (Dk) and joining (JH) segments and an ~200-kb region downstream region harboring the sets of constant region exons (Cyμ, Cβ, Cγ3, Cy1, Cy2β, Cy2a, Cε, and Co) referred as CH genes. Generation of an Ig μ heavy chain protein, which is first expressed in B cell development, involves assembly of a VHJH exon upstream of Cμ via the V(D)J recombination reaction (1). In antigen-stimulated peripheral B cells, expression of downstream IgK light isotypes (such as IgG, IgE, or IgA) can occur via a class switch recombination (CSR) reaction that replaces Cκ with a downstream set of CH exons (2). Somatic hypermutation (SHM) of the variable region exon, which allows selection of higher affinity antibodies, also occurs in antigen-stimulated B cells (3). V(D)J recombination is initiated by the RAG endonuclease, whereas CSR and SHM are initiated by activation induced deaminase (4). The overall mechanisms that control these processes in the context of B cell development and activation are still being elucidated; however, many studies have implicated a role for transcriptional control elements (5). Major known control elements in the IgH locus include the transcriptional enhancer that lies within the intron between JH and Cκ (6), which is referred to as the intronic IgH enhancer (iE), and the set of four enhancers that lie in the 3’ end of the IgH locus are referred to as the 3’ IgH regulatory region (RR) (7). Germ-line promoters flank Vκ segments, D segments, and the sequences (S regions) that mediate CSR (2, 6).

Depending on the mouse strain, there are several hundred or more Vκ segments embedded over several megabases at the 5’ end of the locus, followed by 13 DH segments lying in the 100-kb region just 3’ of the Vκ segments and 4 JH segments, which lie just downstream of the D segments (6). The rearranged VκDJH exon that encodes the variable region of IgH chains is assembled by rearranging IgH 3’ flanking regions in an ordered process. At the pro-B cell stage, Dκ to JH recombination occurs on both IgH alleles, followed by Vκ to DJH joining. A productive VκDJH rearrangement leads to the generation of a μ IgH chain that signals cessation of further Vκ to DJH joining to effect allelic exclusion and development to the pre-B stage at which immunoglobulin light chain (IgL) gene rearrangement occurs (8). Expression of complete IgH/IgL 1g receptor leads to the differentiation of B cells that migrate to the periphery and can be stimulated to undergo CSR and SHM.

Control of V(D)J recombination, in the context of ordered rearrangement and feedback regulation, involves modulating differential accessibility of substrate V, D, and J segments to the RAG endonuclease (1). In this context, accessibility correlates with transcriptional activity of unrearranged (“germ line”) Vκ, D, and JH segments. Before D to JH recombination, germ-line transcription is initiated at a promoter associated with iEμ/Iκ to generate μ transcrpts and a promoter upstream of the DQ52 segment to generate μ0 transcripts (9, 10). Likewise, germ-line VH genes are transcribed in the sense direction from VH promoters before onset of Vκ to DJH rearrangement, and such expression is down-regulated upon expression of a productive μ chain (11). More recently, abundant antisense transcripts of both generic and intergenic VH regions have been described in ref. 12. Although the precise role of germ-line transcription remains unclear, transgenic recombination substrate studies showed that iEμ was necessary and sufficient to activate V(D)J recombination (13), and activation of germ-line promoters leads to chromatin structure changes that confer accessibility (14). However, studies of B cells from chimeric mice generated from ES cells that harbored mutations in which iEμ was deleted showed that deletion of iEμ impaired but did not totally block V(D)J recombination at the JH locus, whereas pgk-Neo replacement of iEμ eliminated such rearrangements, implying redundant IgH cis-acting elements (15–17).

Abbreviations: IgH, immunoglobulin heavy chain; CSR, class switch recombination; SHM, somatic hypermutation; GC, germinal center; RR, regulatory region.

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In addition to being initiated by activation-induced deaminase, both CSR and SHM require transcription through target sequences (S regions and variable region exons) (4). The activity of enhancer elements (i.e., the 3' IgH RR (hS3b and hS4)) is required to activate transcription from promoters flanking S regions (1 promoters) of downstream C_H genes and, thereby, regulates CSR (2, 18). Based on location, iEµ was a candidate to transcriptionally activate Sµ during CSR; correspondingly, deletion of this element appeared to result in a decrease in IgH CSR (19, 20). However, the exact mechanism by which deletion of iEµ affects CSR remains unclear. The region of the IgH and Igk light chain loci targeted for SHM encompasses a 2-kb stretch of DNA that includes the rearranged V_H±DJ_H segment (21–23) with the 5' boundary determined by the V_H or Vk promoter (24–26). The Igk locus also contains an intronic enhancer (iE_k) and a 3' enhancer (3'E_k) (27, 28). Transgenic studies suggested that both iE_k and 3'E_k enhancers were required for SHM (29, 30). However, gene targeted mutation studies showed that the 3'E_k is not required absolutely for SHM, implicating a larger or redundant role for the iE_k (31). Similarly, transgenic studies suggested a role for 3'IgH RR enhancers (hS3b and hS4) for SHM of the IgH locus (32), but gene-targeted mutation showed them to be dispensable (33). However, the role of iEµ in SHM of the endogenous IgH locus has never been tested.

To directly examine the role of iEµ in mediating IgH locus V_H±DJ_H recombinational accessibility, effects on CSR, and potential roles in SHM, we have now generated and analyzed mice in which the cEµ is deleted in the germ line.

Materials and Methods

Gene Targeting. The targeting vector for the IgH Eµ core enhancer element was used to transfect 129 strain TC1 ES cells as described in ref. 17. Two ES cell clones showing homologous replacement of cEµ were injected into C57BL/6 blastocysts, chimeras harboring germ-line mutations mated to 129sv-ev mice to introduce the cEµ replacement mutation (“N” allele) into a pure 129 background. The replacement mutant mice were bred to Eha-cre transgenic mice (34) to generate progeny that deleted the Neo8 cassette by cre-recombined mediation between loxP sites (generating the “∆” allele).

Flow Cytometric Analysis. Single-cell suspensions were prepared, washed, and stained in PBS 2% FCS. For analysis staining, 0.5 × 10^6 cells were incubated at 4°C for 45 min with various monoclonal antibodies conjugated to: phycoerythrin-CyChrome (anti-mouse B220 clone RA3-6B2, BioSource, San Diego), phycoerythrin (anti-mouse CD43 clone S7 and anti-mouse CD4 clone H129.19, BD Biosciences Pharmingen, San Diego) and fluorescein-isothiocyanate (anti-mouse IgMa clone DS1 and anti-mouse CD19 coupled magnetic separation microbeads). The positive fraction was stained with rat monoclonal antibody anti-mouse B220 (clone RA3–6B2) conjugated with phycoerythrin-CyChrome (BioSource), rat monoclonal anti-mouse CD43 (clone S7) conjugated to phycoerythrin, and mouse monoclonal anti-mouse IgMa (clone DS1) conjugated to fluorescein-isothiocyanate (BD Biosciences Pharmingen). The pro-B (IgM– B220+ CD43+) and pre-B (IgM– B220– CD43++) cell fractions were sorted and collected on a FACSinC apparatus (BD Biosciences) and genomic DNA extracted. For Eµ_N/N animals, cells from total bone marrow were isolated and genomic DNA extracted. Genomic DNA was diluted and PCR performed to detect D to J_H and V_H to DJ_H rearrangements (35). Normalization of DNA amount in each diluted sample was estimated by PCR amplification of the IgH locus distal regulatory element hS4 by using HS4–5' and HS4–3’ primers.

RT-PCR Analysis. Eµ_N/N, Eµ∆, and wt mice were backcrossed into a Rag2 null background to obtain double mutant animals. Bone marrow cells were isolated and the CD19+ pro-B cell fraction was purified by magnetic separation as described above. RNA preparation and reverse transcription was performed as described in ref. 12. For quantitative real-time PCR an iCycler (Bio-Rad) was used.

Hybridoma Analysis. Total splenocytes were stimulated with LPS or IL-4/αCD40 in complete RPMI medium 1640 for 4 days and fused to NS-1 plasmacytoma cells. Hybridomas were generated, assessed for clonality, and then analyzed by ELISA for IgH isotype expression in the supernatant and VDJ_H recombination status as described in ref. 35.

Primers. All primers are listed in Table 1, which is published as supporting information on the PNAS web site.

Results

Germ-Line Replacement and Deletion of cEµ. To introduce germ-line cEµ mutations, we used a vector described in ref. 17 that replaces a 220-bp HinfI restriction fragment containing cEµ with a pgk-Neo8 gene flanked by loxP sites (Fig. 6, which is published as supporting information on the PNAS web site). TC1/129 ES cells, and two independent ES cell lines were identified in which the “loxP-Neo8,loxP” cassette correctly replaced cEµ on one IgH allele (cEµ∆ allele). Those clones were injected into C57BL/6 blastocysts and implanted into foster mothers to derive somatic chimeras. Chimeras were bred to either 129sv animals to obtain Eµ_N/N heterozygous mutant animals or with Eha-cre transgenic 129sv mice (34) to obtain animals in which the Neo8 gene was deleted via loxP/Cre-mediated recombination to obtain Eµ_N/∆ mice, which are heterozygous for the “cleanly” deleted cEµ allele. The heterozygous mutant lines were bred, respectively, to obtain Eµ_N/N and Eµ∆∆ animals.

Effects of Eµ_N/N and Eµ∆∆ Mutations on B Cell Development. Previous analyses of cEµ function by targeted mutation used chimeric mice, which can be difficult to assess for subtle developmental defects because of the presence of host B lineage cells. Therefore, we compared B cell development in 8-wk-old germ-
line mutant mice to that of wt counterparts (Fig. 1A–D). Eμ<sup>ΔΔ</sup> animals had small spleens with only 30% of the absolute number of spleenocytes compared to the wild type and lacked identifiable Peyer’s patches and any detectable peripheral B cells (Fig. 1C and data not shown). In addition, Eμ<sup>ΔΔ</sup> mice had an essentially complete arrest of B cell development in the bone marrow at the B220<sup>int</sup> CD43<sup>+</sup> and/or B220<sup>int</sup> cKit<sup>+</sup> Pro-B1 stage where IgH locus V(D)J recombination is initiated (Fig. 1A and B). At 8 wk of age, all Eμ<sup>ΔΔ</sup> animals also exhibited smaller spleens with ~50% the spleenocytes of wt along with a reduced number of mature IgM<sup>+</sup> B cells (Fig. 1C). However, the Eμ<sup>ΔΔ</sup> mice did show normal Peyer’s patches (data not shown). Mature Eμ<sup>ΔΔ</sup> peripheral B cells showed no reduction in surface IgM expression or IgD (Fig. 1E and data not shown). In the bone marrow, there was a more modest impairment of B cell development as compared with the cEμ<sup>NN</sup> blockade, which was evidenced by a significantly increased percentage of B220<sup>int</sup> CD43<sup>+</sup> pro-B cells and a lower percentage of B220<sup>+</sup> CD43<sup>+</sup> pre-B cells (Fig. 1A), again consistent with impairment of V(D)J recombination at the IgH locus. Peripheral and thymic T cells were normal in number and phenotype in both mutant lines (Fig. 1D and data not shown).

**Impaired D<sub>μ</sub> to J<sub>H</sub> and V<sub>μ</sub> to DJ<sub>H</sub> Rearrangement in cEμ<sup>NN</sup> and cEμ<sup>ΔΔ</sup> Mice.** To confirm that the Eμ replacement with a pgk-Neo<sup>R</sup> cassette blocked V(D)J recombination at the IgH locus, we used a PCR-based approach to assay for D to J<sub>H</sub> and V<sub>μ</sub> to DJ<sub>H</sub> rearrangements in DNA from total cEμ<sup>NN</sup> bone marrow and found these rearrangements to be essentially absent (Fig. 7, which is published as supporting information on the PNAS website), demonstrating that the absence of peripheral B cells in these mice corresponds to an essentially complete block of IgH locus V(D)J recombination. A similar PCR analysis of IgH locus V(D)J recombination performed on DNA isolated from sorted cEμ<sup>ΔΔ</sup> pro- and pre-B cells revealed that D-J<sub>H</sub> rearrangements were not markedly impaired as detected by this assay (Fig. 2 Top and Top Middle; also see below). In contrast, cEμ<sup>ΔΔ</sup> pro- and pre-B cells had a substantial decrease in V<sub>μ</sub> to DJ<sub>H</sub> rearrangements (Fig. 2 Top Middle, Bottom Middle, and Bottom). Together, these latter findings support the suggestion that deletion of iEμ has a more major affect on V<sub>μ</sub> to DJ<sub>H</sub> than on D to J<sub>H</sub> rearrangement (17).

To assay more sensitively for potential defects in D to J<sub>H</sub> and V<sub>μ</sub> to DJ<sub>H</sub> rearrangements in cEμ<sup>ΔΔ</sup> B lineage cells at a clonal level, we generated splenic B cell hybridomas from wt and cEμ<sup>ΔΔ</sup> splenic B cells and analyzed the rearrangement status of their J<sub>H</sub> alleles by Southern blotting. Normal B cells generate D to J<sub>H</sub> rearrangements on both J<sub>H</sub> alleles. Correspondingly, we found that only 5% of wt B cell hybridomas contained a germ-line J<sub>H</sub> allele (Fig. 3A), consistent with other studies (17, 35). The occurrence of a small percentage of hybridomas with germ-line alleles is thought to reflect a low level of tripartite fusions with nonlymphoid cells (35). In contrast, ~30% of cEμ<sup>ΔΔ</sup> hybridomas retained the J<sub>H</sub> locus in germ-line configuration on one allele, consistent with defect in D to J<sub>H</sub> rearrangement efficiency (Fig. 3A) that is below the level readily observed by the PCR assay. These hybridoma results that indicated defective D to J<sub>H</sub> rearrangement in the cEμ<sup>ΔΔ</sup> background were confirmed by Southern blotting analysis of LPS-stimulated splenocyte DNA that showed that activated B cells from cEμ<sup>ΔΔ</sup> animals, but not wt animals, retained a detectable level of the 6.2-kb germ-line J<sub>H</sub> hybridizing EcoRI fragment (Fig. 3B). All of the hybridomas must have a productive V<sub>μ</sub>DJ<sub>H</sub> rearrangement. Therefore, to assay for V<sub>μ</sub> to DJ<sub>H</sub> rearrangement defects, we asked what portion of hybridomas that had rearranged both J<sub>H</sub> alleles had V<sub>μ</sub>DJ<sub>H</sub> versus a DJ<sub>H</sub> rearrangement on their nonproductive allele. Normally, ~50–60% of wt B cell hybridomas have the nonproductive J<sub>H</sub> allele in the DJ<sub>H</sub> configuration, and 40–50% have the nonproductive allele in the V<sub>μ</sub>DJ<sub>H</sub> configuration. However, we found that 88% (87 of 99) of cEμ<sup>ΔΔ</sup> hybridomas had the nonproductive allele in the DJ<sub>H</sub> configuration (Fig. 3A), confirming the V<sub>μ</sub> to DJ<sub>H</sub> joining defect indicated by the PCR-based studies with pro-B cells (Figs. 2 and 7).

**Role of Eμ in Germ-Line IgH Transcription.** It was suggested previously that the iEμ regulates μ transcripts, which originate 5′ of...
DQ52 (10), and I\(\mu\) transcripts, which originate immediately 3’ of the E\(\mu\) core (9). To look at expression levels of these transcripts, E\(\mu\)^N\(N\) and E\(\mu\)^\(\Delta\\Delta\) mice were crossed into a Rag2-deficient background. Bone marrow cells were magnetic-activated cell sorted for CD19 surface expression, which allowed us to generate an \(~90\%\) pure pro-B cell population. RT-PCR analysis of E\(\mu\)^\(\Delta\\Delta\) pro-B cells revealed a 10- to 20-fold decrease of I\(\mu\) and \(\mu\)0 transcripts compared with those of wt mice; in these experiments, the level of \(\lambda5\) (Fig. 4A) and Rag1 (Fig. 4B) transcripts, respectively, were used to standardize RNA levels. E\(\mu\)^\(\Delta\\Delta\) pro-B cells also exhibited an approximately 10- to 20-fold decrease in I\(\mu\) transcripts and, strikingly, \(\mu\)0 transcripts were not detectable in these cells by either semiquantitative (Fig. 4A) or quantitative (Fig. 4B) RT-PCR. In contrast to our findings for I\(\mu\) and \(\mu\)0 transcripts, we found that germ-line transcript levels from various E\(\mu\) proximal and distal V\(\mu\) families were similar in E\(\mu\)^N\(N\), E\(\mu\)^\(\Delta\\Delta\), and wt mice (Fig. 4A).

**Reduced Class Switch Recombination in the Absence of the cE\(\mu\).** To investigate IgH class switching in vivo in the absence of the cE\(\mu\) intronic enhancer, we stimulated total splenocytes from wt and E\(\mu\)^\(\Delta\\Delta\) mice, respectively, with LPS (which induces CSR to IgG2b and IgG3) or with cCD40 plus IL-4 (which induces CSR to IgG1) and produced clonal hybridoma lines that were screened for IgH isotype production by ELISA. Analyses of the hybridomas showed that IgH class switching, under either condition, was, at most, modestly diminished (Fig. 8A, which is published as supporting information on the PNAS web site). In this regard, we also quantified, by ELISA, each IgH subclass in the serum of 10-wk-old mutant animals. A cohort of nine cE\(\mu\)^\(\Delta\\Delta\) mice compared with six wt littermates, again revealed, at most, a modest decrease in some, but not all, downstream IgH isotypes (Fig. 8B). Although the defects observed by the above assays were only of borderline significance, it is known that subtle defects in class-switching at the cellular level can become more obvious when CSR is assayed on the second allele of hybridomas that had undergone CSR on one allele (36). In this context, normal B cells undergo CSR on both IgH alleles at very high
frequency (>90%); however, of 29 cEμ/Δλ hybrids analyzed, only 16 (55%) had undergone CSR on the second allele, clearly documenting a role for cEμ in maintaining CSR at its highest efficiency, consistent with earlier findings based on analyses of chimeric mice (19).

**Normal Somatic Hypermutation in cEμ/Δλ B Cells.** Because the cEμ/Δλ mutation allows B cell development to proceed at considerable levels, we were able to assay for the effects of this mutation on SHM of IgH genes. In two independent experiments, comparing three cEμ/Δλ animals and two wt littermates, we PCR amplified and cloned IgH variable region exons and downstream flanking sequences from sorted Peyser’s patch GC B cells. We quantified mutations in the 500-bp region just downstream of the Jμ4 exon and found the mutation frequency in cEμ/Δλ GC B cells to be comparable with that observed in wt controls (Fig. 5A). Thus, when comparing the number of mutations per sequence, we found that cEμ/Δλ GC B cells displayed an approximately normal fraction of sequences that were highly mutated (i.e., with 10 or more mutations in the 500-bp region analyzed; 29% compared with 38% in wt; Fig. 5B). On the other hand, cEμ/Δλ B cells had a 2-fold increase of unmutated sequences (31% in mutants and 14% in wt, Fig. 5B), which might reflect fewer mutant cells entering the GC reaction because of the decreased numbers of peripheral cEμ/Δλ B cells. Finally, we analyzed the pattern of nucleotide substitutions in the absence of the cEμ and found that transitions and transversions occurred about the same frequency at dC/dG or dA/dT (Fig. 5C), suggesting that cEμ is not involved in targeting of particular types of mutations.

**Discussion**

There have been several previous mutational analyses of iEμ function done by an approach in which the targeted Eμ replacements were generated in ES cells, which then were used to analyze the effects of the mutations in the context of mutant B cells generated in chimeric mice (15–17, 19). This approach demonstrated that deletion of cEμ leads to decreased V(D)J recombination at the IgH locus, with the most predominant effect being on VH to DJH rearrangement (15–17), and also demonstrated that cEμ influences IgH class switch recombination (19). Now, we have generated a line of mice that harbor a deletion of cEμ in their germ line. Analyses of these mice confirmed and extended a number of findings made with chimeric mice, including a more detailed analysis of the requirement for iEμ for normal B cell development and confirmation of the existence of an additional element that influences D to JH rearrangement. In addition, our current analyses have provided previously undescribed insights into iEμ function. Thus, we show that the influence of cEμ on V(D)J recombination at the JH locus correlates well with its effects on germ-line transcription through this region. In addition, we have made the unexpected finding that cEμ is not required for SHM of IgH variable region exons.

It was previously shown that deletion of iEμ substantially impairs VH to DJH rearrangement at the IgH locus. We have confirmed this conclusion and clearly documented a significant inhibition of D to JH rearrangement as well. It has been speculated that promoter activity associated with the generation of μ0 transcripts was required for efficient D-JH transcription. The dramatic decrease in μ0 levels observed in cEμ/Δλ pro-B cells suggests that cEμ directly regulates the μ0 promoter and strengthens the hypothesis that this interaction is linked to V(D)J recombinational accessibility of the JH locus. Moreover, our finding that sense germ-line VH transcription appears relatively normal in cEμ/Δλ pro-B raises the possibility that the dramatic influence of cEμ on VH to DJH rearrangement may be mediated via the activity of this element with respect to the D-JH locus versus the VH portion of the IgH locus. For example, the cEμ may be required to activate DJH rearrangement for recombination with upstream VH segments (6). However, another possibility would be an effect on anti-sense VH transcription, which can now be analyzed in the context of the homozygous germ-line iEμ mutation on a RAG-2 deficient background.

In the absence of cEμ, we still observed the development of substantial levels of peripheral B cells, in accord with the observation that cEμ deletion diminishes, but does not block, IgH locus V(D)J recombination. In contrast, replacement of iEμ with a pgk-NeoR gene totally blocked B cell development at the pre-B stage and, correspondingly, completely blocked D to JH rearrangement and assembly of variable region exons (refs. 15–17 and this study). Although the precise mechanism by which replacement of iEμ with a pgk-NeoR gene fully blocks V(D)J recombination remains speculative, we now demonstrate that this mutation, in contrast to the iEμ deletion, fully abrogates expression of μ0 transcripts, most likely due to the effect on transcriptional initiation. Thus, the correlation between the effects on transcription and V(D)J recombination of these two mutations are strongly suggestive of a cause/effect relationship.

**Fig. 5.** Somatic hypermutation of the IgH locus in 129 wt and cEμ/Δλ mice. GC B cell DNA was isolated from B220<sup>+</sup> PNA<sup>hi</sup> Fas<sup>+</sup> Peyer’s patches cells of a nonimmunized 10-wk-old animal. The IgH variable region was amplified as described in ref. 37. (A) Number of mutations versus total length of DNA sequence analyzed, and the mutation frequency in two wt and three mutant animals. Only sequences that contain at least one mutation were included, and cloned related sequences were excluded. (B) Pie plots showing the number of mutations (at the perimeter of the plot) and the percentage of the total number of clones for each percentage group (proportional to the area in each slice); the total number of clones analyzed for each genotype is noted in the center. (C) Pattern of nucleotide substitutions detected in the JH4 intronic region. The table shows the percent of mutations for each type of nucleotide present in the germ-line sequence (vertical axis) to a different nucleotide (horizontal axis). The table below compares the ratio of mutations at dC/dG to those at dA/dT as well as the ratio of transitions: transversions substitutions at both dC/dT as well as the ratio of transitions: transversions substitutions at both dC/dG and dA/dT.
In this context, studies with V(D)J recombination substrates have shown that levels of germ-line transcription correlate precisely with V(D)J recombination efficiency (38) and that promoters have an important role in V(D)J recombination accessibility (14). There are several mechanisms by which the pgk-NeoR replacement might inhibit transcription from the µ promoter (and D to JH rearrangements). One possibility is that the cassette may block µ promoter activation by inhibiting an unknown cis-regulatory element via a promoter competition/insulating mechanism. In this regard, elements in the 3' IgH RR would be attractive candidates because they work over at least 200-kb (39), and their activity has been shown to be inhibited by insertion of a pgk-NeoR cassette between them and their target I region promoters (18, 39–41). Alternatively, pgk-NeoR might alter local chromatin structure and, thereby, interfere with germ-line transcription and D to JH recombination. The availability of RAG-2 deficient mice harboring the iEμ mutations in their germ line will allow the latter possibility to be addressed directly.

It has been shown previously in chimeric mice that deletion of the cEμ somewhat diminishes but does not abrogate CSR (19, 20). Likewise, we have observed only a very modest defect in IgH class switching in cEμΔΔ B cells that was hardly apparent with respect to serum IgH isotype levels but was demonstrable at the IgH locus. Even class switching in cEμΔΔ R cells that was hardly apparent with

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<td>TTCCAAATACCCGAAAGCATTTCAC</td>
<td>53</td>
</tr>
<tr>
<td>(i)_(\mu) Rev</td>
<td>GTCCATGAGCAGCCAGGTTG</td>
<td>53</td>
</tr>
<tr>
<td>(\mu)_(0) For (semi-quantitative PCR)</td>
<td>GGACCTTTGGGCTGCGTTTGG</td>
<td>60</td>
</tr>
<tr>
<td>(\mu)_(0) Rev (semi-quantitative PCR)</td>
<td>CAGCACATGGGGAGACACGTTC</td>
<td>60</td>
</tr>
<tr>
<td>(\mu)_(0) For (quantitative PCR)</td>
<td>GGATCTGCCAGAAACTGAAG</td>
<td>66</td>
</tr>
<tr>
<td>(\mu)_(0) Rev (quantitative PCR)</td>
<td>AGGCTCTGAGATCCCTAGACAG</td>
<td>66</td>
</tr>
<tr>
<td>(\lambda)_(5) For</td>
<td>TCAGCAGAAAAGGAGACAGAGCT</td>
<td>55</td>
</tr>
<tr>
<td>(\lambda)_(5) Rev</td>
<td>ACACACTACGTGAGCCTTTGT</td>
<td>55</td>
</tr>
</tbody>
</table>
### A. Table

<table>
<thead>
<tr>
<th></th>
<th>Total number</th>
<th>IgM</th>
<th>IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>wt</strong></td>
<td>67</td>
<td>58 (87%)</td>
<td>9 (13%)</td>
</tr>
<tr>
<td><strong>cE,μΔ/Δ</strong></td>
<td>116</td>
<td>109 (94%)</td>
<td>7 (6%)</td>
</tr>
<tr>
<td>IL4 αCD40</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>wt</strong></td>
<td>88</td>
<td>40 (45%)</td>
<td>48 (55%)</td>
</tr>
<tr>
<td><strong>cE,μΔ/Δ</strong></td>
<td>107</td>
<td>56 (52%)</td>
<td>51 (48%)</td>
</tr>
</tbody>
</table>

### B. Graph

- IgM
- IgG3
- IgG1
- IgG2b
- IgG2a
- IgE
- IgA

**X-axis:** Genotype (WT, AΔ, NIN)

**Y-axis:** Serum Ig [μg/mL]

- Range from 0.1 to 10,000 μg/mL