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 (iEμ) 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 iEμ (cEμ) were either deleted (cEμΔα mice) or replaced with a pgk-Neo® cassette (cEμNM mice). The cEμΔα mice had reduced B cell numbers, in association with impaired D to JH and VH to DJH rearrangement, whereas cEμNM mice had a complete block in IgH V(D)Jμ recombination, confirming that additional cis elements cooperate with iEμ to enforce D to JH recombination. In addition, developing cEμHΔα and cEμHNM B lineage cells had correspondingly decreased levels of germ-line transcripts from the JH region of the IgH locus (μ0 and Iα transcripts); although both had normal levels of germ-line Vα transcripts, suggesting that cEμ may influence IgH locus V(D)Jμ recombination by influencing accessibility of Iα proximal regions of the locus. Consistent with chimeric studies, peripheral cEμΔα B cells had normal surface Ig and relatively normal class switch recombination. However, cEμΔα and cEμHΔα cells also had relatively normal somatic hypermutation of their IgH variable region genes, showing unexpectedly that the cEμ is not required for this process. The availability of mice with the iEμ mutation in their germ line will facilitate future studies to elucidate the roles of iEμ in V(D)Jμ 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 (Dμ) and joining (JH) segments, and a ∼200-kb region downstream harboring the sets of constant region exons (Cμ, Cδ, Cε3, Cγ1, Cγ2b, Cγ2a, Cε, and Co) referred to as Cμ genes. Generation of an Ig μ heavy chain protein, which is first expressed in B cell development, involves assembly of a VμDJH exon upstream of Cμ via the V(D)J recombination reaction (1). In antigen-stimulated peripheral B cells, expression of downstream IgH isoforms (such as IgG, IgE, or IgA) can occur via a class switch recombination (CSR) reaction that replaces Cα with a downstream set of CH1 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 JHα and Cα (6), which is referred to as the intrinsic IgH enhancer (IEμ), 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 Vμ segments, D segments, and the sequences (S regions) that mediate CSR (2, 6).

Depending on the mouse strain, there are several hundred or more Vμ segments embedded over several megabases at the 5′ end of the locus, followed by 13 Dμ segments lying in the 100-kb region just 3′ of the Vμ segments and 4 JH segments, which lie just downstream of the D segments (6). The rearranged VμDJH exon that encodes the variable region of IgH chains is assembled by randomizing the B lineage before an ordered process. At the pro-B cell stage, Dμ to JH recombination occurs on both IgH alleles, followed by Vμ to DJH joining. A productive VμDJH rearrangement leads to the generation of a μ IgH chain that signals cessation of further Vμ 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 Ig 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 unarranged ("germ line") VH, D, and JH segments. Before D to JH rearrangement, germ-line transcription is initiated at a promoter associated with iEμ/Iα to generate Iα transcripts 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 Vμ to DJH rearrangement, and such expression is down-regulated upon expression of a productive μ chain (11). More recently, abundant antisense transcripts of both genic 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 diminished 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 within the 3' IgH RR (hs3b and hs4) is required to activate transcription from promoters flanking S regions (I promoters) of downstream CH 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 Igκ light chain loci targeted for SHM encompasses a 2-kb stretch of DNA that includes the rearranged VμDJH segment (21–23) with the 5' boundary determined by the VH or Vk promoter (24–26). The Igκ locus also contains an intronic enhancer (iEκ) and a 3' enhancer (3'Eκ) (27, 28). Transgenic studies suggested that both iEκ and 3'Eκ enhancers were required for SHM (29, 30). However, gene targeted mutation studies showed that the 3'Eκ is not required absolutely for SHM, implicating a larger or redundant role for the iEκ (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 VμDJH recombinatorial 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 Eμa-cre transgenic mice (34) to generate progeny that deleted the Neoα cassette by cre-mediated recombination betweenloxP sites (generating the “Δ” allele).

Flow Cytometric Analysis. Single-cell suspensions were prepared, washed, and stained in PBS 2% FCS. For analysis staining, 0.5 × 10⁶ cells were incubated at 4°C for 45 min with various monoclonal antibodies conjugated to: phycoerythrin-CyChrome (anti-mouse B220 clone RA3-6B2, Eβioscience, San Diego), phycoerythrin (anti-mouse CD43 clone S7 and anti-mouse CD4 clone Perlot et al., eBioscience, San Diego), phycoerythrin-CyChrome (anti-mouse B220 clone RA3-6B2, eBioscience, San Diego), phycoerythrin (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 (Eβioscience), 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+CD43hi) and pre-B (IgM+ B220+CD43low) cell fractions were sorted and collected on a FACSVantage apparatus (BD Biosciences) and genomic DNA extracted. For EμN/N animals, cells from total bone marrow were isolated and genomic DNA extracted. Genomic DNA was diluted and PCR performed to detect D to JH and VH to DJH rearrangements (35).

Somatic Hypermutation Assays. Peyer’s patch cells from wt and EμN/N mice were stained in 1.5 ml of PBS/2% FCS with rat monoclonal anti-mouse B220 clone RA3-6B2 conjugated with phycoerythrin-CyChrome (Eβioscience); peanut agglutinin (PNA) conjugated to fluorescein-isothiocyanate (Vector Laboratories, Burlingame, CA) and hamster anti-mouse FAS (clone Jo2) conjugated to phycoerythrin (BD Biosciences Pharmingen); and germinal center (GC) B cells (B220+ FAS+ PNAβ+) were sorted and collected on a FACSVantage (BD Biosciences), genomic DNA was extracted and the VμJ558 to JH intronic region was amplified by using high-fidelity Pfu polymerase (BD Biosciences Stratagene, San Diego), the 700-bp products were cloned into the pCR4Blunt-TOPO vector (Invitrogen, Paisley, U.K.), and sequences were analyzed via LASERGENE SEQUAN II software (DNASTAR, Madison, WI).

ELISA Assays for CSR. Sera from 10-week-old EμN/N, EμΔΔ, and wt mice were analyzed for the presence of different IgH classes and subclasses by ELISA (18). Each ELISA titration was performed twice.

Assays for VDJμ and DJH Rearrangements. For EμΔΔ animals, bone marrow cells were isolated and B cells purified by positive magnetic sort on LS columns-MidiMacs (magnetic cell sorting, Miltenyi Biotec, Bergisch Gladbach, Germany) after labeling with 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 (Eβioscience), 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+CD43hi) and pre-B (IgM+ B220+CD43low) cell fractions were sorted and collected on a FACSVantage apparatus (BD Biosciences) and genomic DNA extracted. For EμN/N animals, cells from total bone marrow were isolated and genomic DNA extracted. Genomic DNA was diluted and PCR performed to detect D to JH and VH to DJH rearrangements (35).

RT-PCR Analysis. EμN/N, EμΔΔ, and wt mice were backcrossed into a Rag2−/− 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 VDJH 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-Neoα gene flanked byloxP sites (Fig. 6, which is published as supporting information on the PNAS web site). TC1/129 ES cells were targeted, and two independent ES cell lines were identified in which the “loxP-NeoαloxP” cassette correctly replaced cEμ on one IgH allele (cEμα allele). Those clones were injected into C57/BL6 blastocysts and implanted into foster mothers to derive somatic chimeras. Chimeras were bred to either 129sv animals to obtain Eμαα heterozygous mutant animals or with Eμa-cre transgenic 129sv mice (34) to obtain animals in which the Neoα gene was deleted vialoxP/Cre-mediated recombination to obtain cEμαΔΔ mice, which are heterozygous for the “cleanly” deleted cEμ allele. The heterozygous mutant lines were bred, respectively, to obtain cEμN/N and cEμΔΔ animals.

Effects of EμN/N and EμΔΔ 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 splenocytes 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> ProB1 stage where IgH locus V(D)J recombination is initiated (Fig. 1A and B). At 8 wk of age, all cEμ<sup>ΔΔ</sup> animals also exhibited smaller spleens with ~50% the splenocytes 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 cEμ<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>i</sub> to J<sub>π</sub> and V<sub>j</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>j</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>j</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>j</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>j</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>j</sub> to DJ<sub>H</sub> rearrangement. Therefore, to assay for V<sub>j</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>j</sub> to 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>j</sub> to 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>j</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

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**Fig. 1.** Flow cytometric analysis of B cell lineage populations. (A) FACS analysis of bone marrow cells from tibias and femurs of 129 wt, cEμ<sup>NN</sup>, and cEμ<sup>ΔΔ</sup> animals. Cells were stained with CyC anti-B220, FITC anti-IgMa, and PE anti-CD43 antibodies and gated on the IgM<sup>+</sup> population. B220<sup>int</sup> CD43<sup>+</sup> pro-B and B220<sup>+</sup> CD43<sup>+</sup> pre-B cell populations are shown. (B) FACS analysis of bone marrow stained with CyC anti-B220, FITC anti-IgMa, and PE anti-CD43 antibodies and gated on the IgM<sup>+</sup> population. B220<sup>int</sup> CD43<sup>+</sup> pro-B cell population is shown. (C) FACS analysis of spleen cells stained with CyC anti-B220, FITC anti-IgMa antibodies: B220<sup>+</sup> IgM<sup>+</sup> mature B cell population is shown. (D) FACS analysis of spleen cells stained with CyC anti-CD3, FITC anti-CD4, and PE anti-CD8 antibodies: CD4<sup>+</sup> and CD8<sup>+</sup> T cell populations are shown. (E) Comparative IgM surface expression of B220<sup>+</sup> splenocytes from cEμ<sup>ΔΔ</sup> and 129 wt animals. Mice were analyzed at 4–6 wk of age.
pro-B and pre-B cell populations from 129 wt and cE mice were amplified, and produced clonal hybridoma lines that were screened for decrease in Ig isotype production by ELISA. Analyses of the hybridomas showed that modestly diminished (Fig. 8A) IgH transcription levels compared with those of wt mice; in these experiments, the level of IgH intronic enhancer, we stimulated total splenocytes from wt and cE mice, respectively, were used to standardize RNA levels. IgH transcripts, respectively, were used to normalize DNA input. Mice were analyzed at 6 wk of age.

Fig. 2. IgH D-JH and V<sub>μ</sub>-D<sub>μ</sub> rearrangement assays. Genomic DNA from sorted pro-B and pre-B cell populations from 129 wt and cE mice was subjected to PCR amplification of D<sub>H</sub> to J<sub>H</sub> and V<sub>μ</sub> to D<sub>μ</sub> rearrangements. PCR reactions were performed on serial 3-fold dilutions and products detected via Southern blot hybridization by using an oligonucleotide probe specific to J<sub>H</sub>. Kidney DNA was a negative control. Another independent PCR assay amplifying the 3' IgH regulatory hs4 enhancer was performed to normalize DNA input. Mice were subjected to PCR analysis to detect the indicated transcripts, serial dilutions were 10-fold, and products detected by Southern blotting with a 0.47-kb HindIII-NaeI fragment containing the J<sub>H</sub>4 segment. On the same blot, DNA normalization was controlled by hybridization to a 0.8-kb EcoR1-StuI fragment specific of the germ-line configuration was detected by Southern blotting of a 0.8-kb HindII-Nael fragment containing the J<sub>H</sub>4 segment. Reduced Class Switch Recombination in the Absence of the cE<sub>μ</sub> core (9). To look at expression levels of these transcripts, E<sub>μ</sub><sup>NN</sup> and E<sub>μ</sub><sup>Δ</sup> 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<sub>μ</sub><sup>Δ</sup>-pro-B cells revealed a 10- to 20-fold decrease of I<sub>μ</sub> transcripts, which originate immediately 3' from the E<sub>μ</sub> core (9). To look at expression levels of these transcripts, E<sub>μ</sub><sup>Δ</sup>-pro-B cells also exhibited an approximately 10- to 20-fold decrease in I<sub>μ</sub> transcripts and, strikingly, μ0 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<sub>μ</sub> and μ0 transcripts, we found that germ-line transcript levels from various E<sub>μ</sub> proximal and distal V<sub>H</sub> families were similar in E<sub>μ</sub><sup>NN</sup>, E<sub>μ</sub><sup>Δ</sup>, and wt mice (Fig. 4A).

Fig. 3. IgH rearrangement status of 129 wt and cE<sub>μ</sub><sup>Δ</sup>-peripheral B cells. (A) V<sub>V</sub>D<sub>J</sub>H<sub>H</sub> rearrangement status of hybridomas obtained after fusion of splenic wt and cE<sub>μ</sub><sup>Δ</sup> B cells. Rearrangement status was assayed by Southern blotting (35). Results of three independent fusions are shown. (B) Rearrangement status of nonproductive IgH alleles in peripheral B cells. DNA prepared from positively sorted splenic B cells was digested with EcoR1 and the 6.2-kb restriction fragment corresponding to the germ-line configuration was detected by Southern blotting with a 0.47-kb HindIII-Nael fragment containing the J<sub>H</sub>4 segment. The observed defects in CSR are most 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 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<sub>μ</sub><sup>Δ</sup>-mutant mice were bred 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 cEµΔ/Δ hybridomas 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 cEµΔ/Δ B Cells. Because the cEµΔ/Δ 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 cEµΔ/Δ animals and two wt littermates, we PCR amplified and cloned IgH variable region exons and downstream flanking sequences from sorted Peyer’s patch GC B cells. We quantified mutations in the 500-bp region just downstream of the JH4 exon and found the mutation frequency in cEµΔ/Δ 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 cEµΔ/Δ 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, cEµΔ/Δ 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 cEµΔ/Δ 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 impaired 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 cEµΔ/Δ 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 cEµΔ/Δ 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 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 pro-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.
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-Neo⁸ replacement might inhibit transcription from the µ promoter (and D to IgH 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-Neo⁸ cassette between them and their target I region promoters (18, 39–41). Alternatively, pgk-Neo⁸ might alter local chromatin structure and, thereby, interfere with germ-line transcription and D to IgH recombination. The availability of RAG-2 deficient mice harboring the iE IgH regulatory region elements or other unknown elements cannot be excluded. These findings are reminiscent of findings that deletion of the intronic µκ light chain locus enhancer also does not dramatically affect expression of this locus (42), whereas deletion of the 3'Eκ does (43). Finally, we have shown that cEμ is dispensable for substantial levels of SHM of rearranged IgH variable region exons. In this context, our findings also imply, perhaps surprisingly given findings from transgenic studies (23, 44, 45), that cEμ is not necessary to target the SHM machinery to IgH variable region exons, although additional studies will be necessary to determine whether there is any alteration in the overall DNA sequences targeted. In any case, these results indicate that other elements must function, at least in part, to fulfill the SHM targeting function at the IgH locus.

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