

# Critical roles of the immunoglobulin intronic enhancers in maintaining the sequential rearrangement of *IgH* and *Igk* loci

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**V(D)J recombination of immunoglobulin (Ig) heavy (*IgH*) and light chain genes occurs sequentially in the pro- and pre-B cells. To identify cis-elements that dictate this order of rearrangement, we replaced the endogenous matrix attachment region/*Igk* intronic enhancer (MiE<sub>κ</sub>) with its heavy chain counterpart (E<sub>μ</sub>) in mice. This replacement, denoted E<sub>μ</sub>R, substantially increases the accessibility of both V<sub>κ</sub> and J<sub>κ</sub> loci to V(D)J recombinase in pro-B cells and induces *Igk* rearrangement in these cells. However, E<sub>μ</sub>R does not support *Igk* rearrangement in pre-B cells. Similar to that in MiE<sub>κ</sub><sup>-/-</sup> pre-B cells, the accessibility of V<sub>κ</sub> segments to V(D)J recombinase is considerably reduced in E<sub>μ</sub>R pre-B cells when compared with wild-type pre-B cells. Therefore, E<sub>μ</sub> and MiE<sub>κ</sub> play developmental stage-specific roles in maintaining the sequential rearrangement of *IgH* and *Igk* loci by promoting the accessibility of V, D, and J loci to the V(D)J recombinase.**

## CORRESPONDENCE

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Abbreviations used: ChIP, chromatin immunoprecipitation; ES, embryonic stem; GT, germline transcript; hC<sub>κ</sub>, human κ constant region; LM, ligation mediated; MAR, matrix attachment region; mC<sub>κ</sub>, mouse κ constant region; MiE<sub>κ</sub>, MAR/*Igk* intronic enhancer; MSRE-QPCR, methylation-sensitive restriction enzyme real-time quantitative PCR; PGK, phosphoglycerol kinase; RS, recombining sequence; RSS, recombination signal sequence; SE, signal end.

The Ig and TCR genes are assembled through the somatic recombination of V (Variable), D (Diversity), and J (Junction) gene segments during the early stages of lymphocyte development (1). B lymphocytes express one set of the heavy chain (*IgH*) and either the κ or λ light chain loci (*IgL*). V(D)J recombination of the *IgH* and *IgL* chain genes occurs in a sequential manner. *IgH* rearrangement occurs primarily in pro-B cells, and *IgL* rearrangement occurs primarily in pre-B cells (1). The accessibility of each antigen receptor locus to the recombination machinery regulates its timing and efficiency of rearrangement (2–4). During the appropriate developmental stage, chromatin remodeling complexes modify the chromatin structure of the locus and convert it into a state accessible to the V(D)J recombinase (5–7). Such changes in accessibility are thought to be mediated by cis-elements residing within each locus (5).

Both *IgH* and *Igk* loci contain an enhancer between the J segments and the constant region, which are called *IgH* intronic enhancer (E<sub>μ</sub>) and matrix attachment region (MAR)/*Igk* intronic enhancer (MiE<sub>κ</sub>), respectively (8–12). Both loci also contain multiple other regulatory elements downstream or within their constant region exons. The *Igk* locus contains the

3'E<sub>κ</sub> (13) and Ed (14) enhancers, and the *IgH* locus contains four enhancer elements in its 3'E<sub>H</sub> regulatory region (hs3a, hs1/2, hs3b, and hs4; references 15–17). The deletion of E<sub>μ</sub> in mice severely decreases V<sub>H</sub> to DJ<sub>H</sub> rearrangement (18–20). The deletion of either MiE<sub>κ</sub> or 3'E<sub>κ</sub> from the endogenous *Igk* locus differentially decreases the rearrangement of *Igk* (21, 22). In addition, the deletion of both *Igk* enhancers abolishes *Igk* rearrangement, indicating that these two enhancers play redundant and essential roles in activating *Igk* rearrangement (23).

The mechanisms by which cis-elements activate *Igk* recombination remain unclear. One potential mechanism is through the activation of germline transcription from germline promoters located upstream of J<sub>κ</sub> (24–27), as κ<sup>0</sup>GT [germline transcription] appears to play a quantitative role in activating *Igk* rearrangement (28, 29). Germline transcription of V<sub>κ</sub> gene segments (V<sub>κ</sub>GT) may also play a role in regulating the accessibility of the V<sub>κ</sub> regions (30). Another mechanism could be DNA demethylation (31). In this context, DNA methylation is inversely correlated with the efficiency of *Igk* rearrangement, and *Igk* enhancers are critical for demethylation of the *Igk* locus (23). However, only a minor demethylation of *Igk* can be detected in WT pre-B cells (32), suggesting that demethylation occurs within a small pre-B cell

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population immediately before the rearrangement of this locus. Hypomethylation of the *Igk* locus resulting from the targeted deletion of the methyltransferase gene *Dnmt1* is not sufficient to activate *Igk* rearrangement in pre-B cell lines, suggesting that DNA demethylation alone is not sufficient in activating *Igk* rearrangement (33).

Recent studies suggest that histone modification is involved in regulating the accessibility to V(D)J recombinase. Acetylation of the H3 and H4 histones spanning the  $D_H$ - $J_H$  regions occurs during  $D_H$  to  $J_H$  rearrangement, and acetylation of  $V_H$  histones occurs during  $V_H$  to  $D_JH$  rearrangement (34). However,  $V_H$  histones are deacetylated during the transition to the pre-B cell stage, in part as a result of a loss of IL-7 signaling (35). H3 and H4 acetylation at the *Igk* locus appears to originate at the pro-B cell stage (36, 37) but increases substantially in pre-B cells (37). Enhancer elements may contribute to the chromatin remodeling events by recruiting histone modifiers to the Ig loci (38). Overexpression of E2A is sufficient to activate *Igk* rearrangement likely through its direct binding to the *Igk* intronic enhancer (39, 40) and  $V_K$  promoters (41). In addition, E2A has been shown to interact with multiple histone acetyltransferase complexes (42–44).

Because enhancers are important in activating V(D)J rearrangement, we hypothesized that  $E\mu$  might be involved in this developmental stage-specific process. To test this hypothesis, we replaced the endogenous  $MiE_K$  with  $E\mu$  in mice.

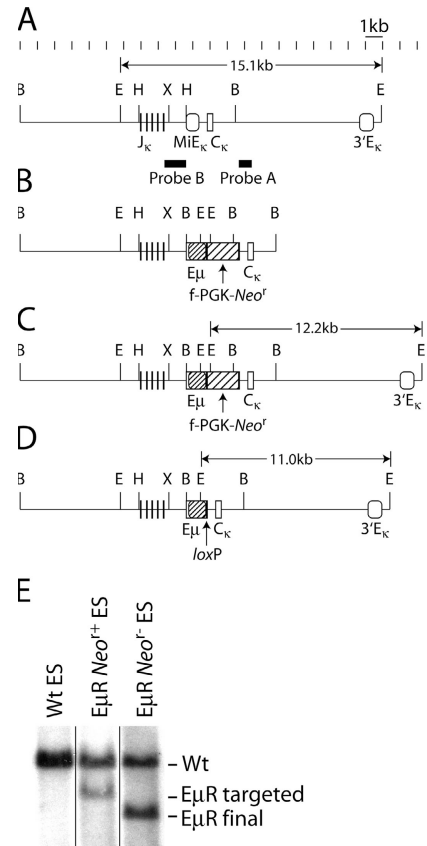
## RESULTS

### Generation of $E\mu$ R mice

To replace the endogenous *Igk* intronic enhancer with the heavy chain intronic enhancer ( $E\mu$ ), the 0.8-kb *Xba*I fragment spanning the entire  $E\mu$  element and flanking MAR sequences was inserted 5' of a *loxP*-flanked phosphoglycerol kinase (PGK)-*Neo<sup>r</sup>* gene (*f-PGK-Neo<sup>r</sup>*). This enhancer replacement cassette was placed into a targeting vector that was used previously to delete *Igk* intronic enhancer (Fig. 1 B; reference 21). Homologous recombination was confirmed by Southern blotting with *Eco*RI digestion of genomic DNA and hybridization to probe A (Fig. 1, A, C, and E). To remove the neomycin resistance gene from the targeted allele, heterozygous mutant embryonic stem (ES) cells were transiently transfected with a plasmid that drives the expression of the *Cre* recombinase. Deletion of the *Neo<sup>r</sup>* gene in subcloned ES cells was confirmed by Southern blotting with *Eco*RI digestion and hybridization to probe A (Fig. 1, A, D, and E). *PGK-Neo<sup>r</sup>*-deleted ES clones were used to generate chimeric animals that transmitted the  $E\mu$ R mutation into mouse germline.

### $E\mu$ R mice exhibit moderate defects in $\kappa^+$ B cell development

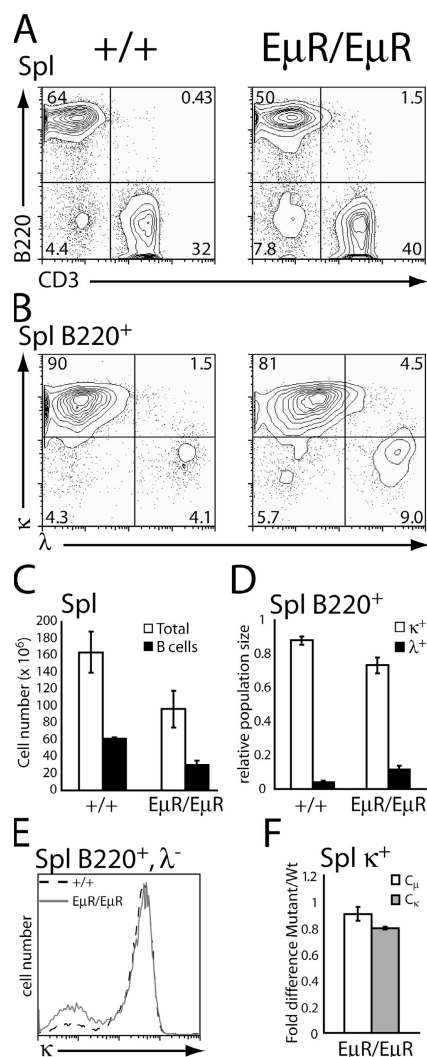
To determine the effects of the  $E\mu$ R mutation on B cell development, BM and spleen cells derived from WT and  $E\mu$ R mice were analyzed by flow cytometry. In spleens from  $E\mu$ R mice, we found an  $\sim$ 50% decrease in the total number of B cells compared with WT controls (Fig. 2 C). The percentage of  $\kappa^+$  splenic B cells was reduced, lowering the  $\kappa/\lambda$  ratio from 20:1



**Figure 1. Generation of  $E\mu$ R knockin ES cells and mice.** (A) Endogenous germline *Igk* locus. The lengths of the diagnostic restriction fragments and probes are shown. Markings above the diagram are spaced at 1-kb intervals. All maps are to scale. (B) Targeting construct to replace  $MiE_K$  with  $E\mu$  (striped rounded box) and floxed PGK-*Neo<sup>r</sup>* (*f-PGK-Neo<sup>r</sup>*; striped box). (C) Targeted locus with *f-PGK-Neo<sup>r</sup>* inserted. The size of the mutant *Eco*RI restriction fragment is shown. (D) Knockin allele with *f-PGK-Neo<sup>r</sup>* removed by *Cre/loxP*-mediated deletion. (E) Southern blotting analysis of ES cell genomic DNA digested with *Eco*RI and probed with probe A. First lane, WT ES cell DNA; second lane, heterozygous  $E\mu$ R ES cell with *PGK-Neo<sup>r</sup>* inserted; third lane, heterozygous  $E\mu$ R knockin with *PGK-Neo<sup>r</sup>* deleted. Bands corresponding to the WT,  $E\mu$ R with *PGK-Neo<sup>r</sup>* inserted, and knockin alleles are indicated on the right. B, BamHI; E, *Eco*RI; H, HindIII; X, *Xba*I.

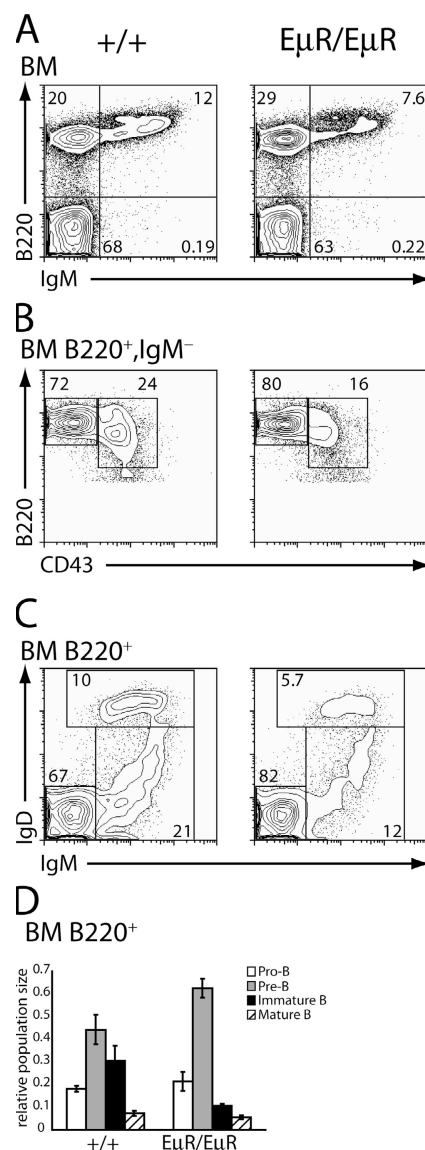
in WT mice to 6:1 in  $E\mu$ R mice (Fig. 2, B and D). These data indicate an impairment in the development of  $\kappa^+$  B cells. This defect is not caused by impaired *Igk* expression because both the surface expression and transcription of *Igk* were identical between WT and  $E\mu$ R splenic  $\kappa^+$  B cells (Fig. 2, E and F).

Consistent with the reduction of B cells in the spleen, a moderate increase in the percentage of pre-B cells ( $B220^+CD43^-IgM^-$ ) and a decrease in immature B cells ( $B220^+IgM^+IgD^{low}$ ) was detected in  $E\mu$ R BM compared with those in WT controls (Fig. 3, A–C). Because the percentage of pro-B cells was similar between WT and  $E\mu$ R BM, these data indicate that the defect in  $E\mu$ R B cell development originates at the pre-B cell stage, where *Igk*



**Figure 2. Analysis of splenic B cells in WT and  $E\mu R$  5–8-wk-old littermates.** (A) Flow cytometric analysis of B cells (B220<sup>+</sup>) and T cells (CD3<sup>+</sup>) in the spleens of WT and  $E\mu R$  mice. The genotypes are indicated at the top. Live cells (propidium iodide<sup>-</sup>) within the lymphoid gate are shown. (B)  $\kappa/\lambda$  ratio of splenic B220<sup>+</sup> cells. (A and B) Percentages of total cells within each quadrant are shown. (C) B cell counts in 1-mo-old WT and  $E\mu R$  spleens. Absolute numbers of total white blood cells (white bars) and B cells (black bars) are shown with error bars (SD;  $n = 4$ ). (D) Proportions of splenic  $\kappa^+$  (white bars) and  $\lambda^+$  (black bars) B cells shown with error bars (SD;  $n = 5$ ). (E)  $\kappa$  surface expression in WT (dashed line) and  $E\mu R$  (solid gray line) B220<sup>+</sup>,  $\lambda^-$  splenic lymphocytes. (F) Analysis of  $\kappa$  mRNA levels in  $\kappa^+$  splenic B cells by quantitative real-time PCR. The  $\kappa$  mRNA levels were normalized to GAPDH mRNA levels. The ratio of mRNA levels of  $C_{\mu}$  and  $C_{\kappa}$  in  $E\mu R$  B cells relative to WT are shown with error bars (SD;  $n = 2$ ).

normally rearranges (Fig. 3 D). This partial developmental block is similar to that observed in  $MiE_{\kappa}^{-/-}$  mice (Fig. S1, A, B, and E; available at <http://www.jem.org/cgi/content/full/jem.20052310/DC1>). However,  $MiE_{\kappa}^{-/-}$  mice have a much more severe decrease in the  $\kappa/\lambda$  ratio in splenic B cells than that observed in  $E\mu R$  mice (Fig. S1, B and F).



**Figure 3. Analysis of BM B cell populations.** (A) Flow cytometric analysis of BM from WT and  $E\mu R$  mice. Percentages of total cells within each quadrant are shown. Genotypes are indicated above each blot. (B) BM pro- and pre-B cell population. Only B220<sup>+</sup>, IgM<sup>-</sup> cells within the lymphocyte gate are shown. (C) BM cells were stained with anti-B220, anti-IgM, and anti-IgD antibodies. Only B220<sup>+</sup> cells are shown. Gates for pro-/pre-B cells (B220<sup>+</sup>/IgM<sup>-</sup>/IgD<sup>-</sup>), immature B cells (B220<sup>+</sup>/IgM<sup>+</sup>/IgD<sup>-/lo</sup>), and mature B cells (B220<sup>+</sup>/IgM<sup>+</sup>/IgD<sup>hi</sup>) are indicated. The percentages of total cells within the lymphoid gate are shown. (D) Statistical analysis of the populations of B lineage cells in the BM of 5–8-wk-old WT and  $E\mu R$  littermates (error bars represent SD;  $n = 2$ ).

To directly compare the overall rearrangement efficiency of the  $E\mu R$  and WT allele, we bred  $E\mu R$  mice with knockin mice with their mouse  $\kappa$  constant region ( $mC_{\kappa}$ ) replaced with the human  $\kappa$  constant region ( $hC_{\kappa}$ ; Fig. S2 A, available at <http://www.jem.org/cgi/content/full/jem.20052310/DC1>). Analysis of  $+/hC_{\kappa}$  and  $E\mu R/hC_{\kappa}$  mice showed that they had similar populations of B cells in BM

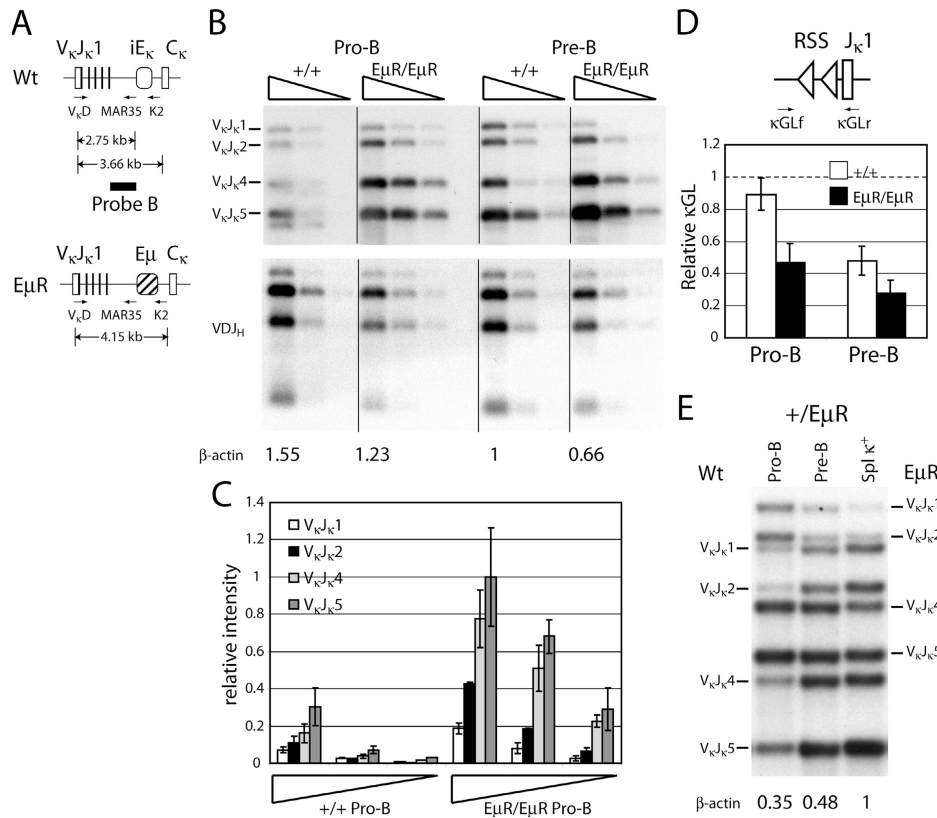
(Fig. S2 B). In the spleen, +/hC<sub>κ</sub> mice had approximately equal numbers of hC<sub>κ</sub><sup>+</sup> and mC<sub>κ</sub><sup>+</sup> B cells (Fig. S2, C and D). However, EμR/hC<sub>κ</sub> mice had a dramatic reduction in the percentage of mC<sub>κ</sub><sup>+</sup> B cells with a ratio of hC<sub>κ</sub><sup>+</sup>/mC<sub>κ</sub><sup>+</sup> B cells of ~6:1 (Fig. S2, C and D). Therefore, the overall efficiency of the EμR allele appears to be lower than that of the WT allele.

**Igk rearrangement is activated by EμR in pro-B cells**

To examine whether EμR affects the timing of V(D)J recombination, we used a PCR assay to analyze *Igk* rearrangement in WT and EμR homozygous pro- and pre-B cells (Fig. 4 A). The degenerate V<sub>κ</sub> primer (V<sub>κ</sub>D), which binds to ~90% of V<sub>κ</sub> gene segments, was used together with a primer

downstream of J<sub>κ</sub>5 (primer MAR35; reference 26). Consistent with previous studies, a very low level of *Igk* rearrangement was detected in WT pro-B cells (Fig. 4 B, left; references 45, 46). However, rearrangement of the EμR allele is dramatically increased (~16-fold) in the pro-B cells of EμR mice (Fig. 4, B and C). At the pre-B cell stage, the levels of *Igk* rearrangement were similar between the WT and EμR mice (Fig. 4 B, right).

To estimate the level of rearrangement of EμR allele in pro-B cells, we designed a real-time PCR assay using primers that flanked the J<sub>κ</sub>1 gene segment and its recombination signal sequence (RSS) to analyze the level of unrearranged *Igk* allele in pro-B cells (Fig. 4 D, top). As this PCR reaction cannot amplify the majority of rearranged *Igk* alleles in pre-B



**Figure 4. *Igk* rearrangement in +/+, EμR/EμR, and +/EμR mice.** (A) PCR strategy to detect *Igk* rearrangement. Configuration of the rearranged *Igk* locus in WT and EμR B lineage cells. Location of the PCR primers and probe and the size of the PCR product for a V<sub>κ</sub> to J<sub>κ</sub>1 rearrangement are shown. (B) Semiquantitative PCR analysis of *Igk* rearrangement using primers V<sub>κ</sub>D and MAR35 in the pro- and pre-B cells of WT and EμR mice. Genomic DNA was serially diluted fourfold. Bands corresponding to the rearrangement of V<sub>κ</sub> to each of the four J<sub>κ</sub> gene segments are indicated on the left. To control for the amount of genomic DNA used for PCR analysis, rearrangement of the *IgH* locus (VDJ<sub>H</sub>) is shown in the bottom panel. The relative amount of genomic DNA used for the PCR reaction was determined by quantitative real-time PCR analysis of the β-actin gene and is shown at the bottom. (C) Quantitative analysis of *Igk* rearrangement in pro-B cells. The intensity of each ampli-

fied product of V<sub>κ</sub>J<sub>κ</sub> rearrangement is normalized to the intensity of VDJ<sub>H</sub> rearrangement. (D) Percentage of unrearranged *Igk* alleles (κGL) in WT and EμR pro- and pre-B cells. The primer annealing locations for the forward (κGLf) and reverse (κGLr) primers are shown. κGL levels were normalized to the levels of the β-actin genomic region. The percentage is calculated by dividing the κGL levels in +/+ or EμR/EμR pre-B cells by those in ES cells. Error bars represent SD. (E) PCR amplification of rearranged *Igk* alleles in sorted heterozygous (+/EμR) B lineage cells. Genomic DNA from sorted pro-, pre-, and mature B cells was amplified with the degenerate V<sub>κ</sub> primer and primer K2. Bands corresponding to the rearrangements of V<sub>κ</sub> to each of the four J<sub>κ</sub> gene segments of the WT allele and EμR allele are indicated. The relative amount of genomic DNA used for the PCR reaction is shown at the bottom.



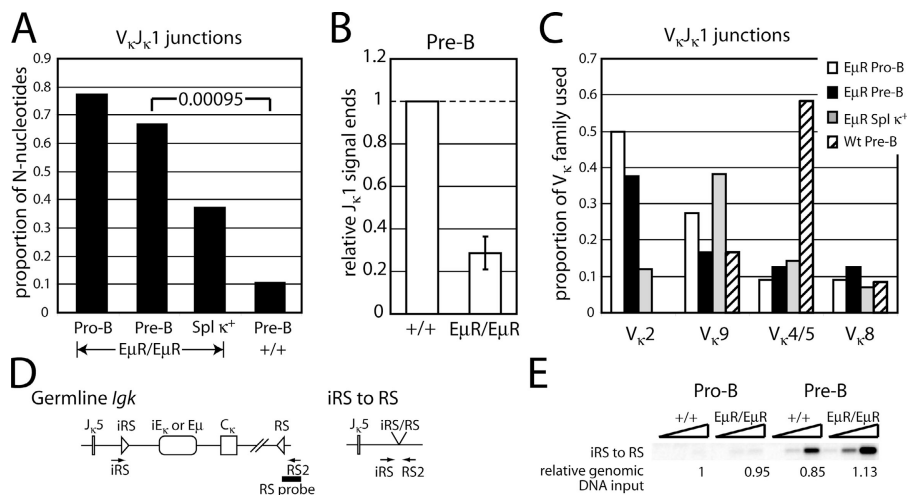
cells, it allowed us to estimate the proportion of unrearranged *Igk* loci using genomic DNA from the nonrearranging ES cells as a control. Based on this assay, it was estimated that ~90 and 50% of *Igk* alleles were unrearranged in WT and  $E\mu R$  pro-B cells, respectively, indicating substantial *Igk* rearrangement in  $E\mu R$  pro-B cells (Fig. 4 D). In addition, it was estimated that ~50% of WT *Igk* alleles were unrearranged in pre-B cells, whereas ~30% of  $E\mu R$  alleles were unrearranged (Fig. 4 D).

To further compare *Igk* rearrangement efficiency between the WT and  $E\mu R$  alleles, we analyzed *Igk* rearrangement in pro-, pre-, and mature  $\kappa^+$  B cells derived from heterozygous (+/ $E\mu R$ ) mice using primers  $V_{\kappa}D$  and K2 (Fig. 4 E). The small size difference between the PCR products amplified from the rearranged WT and  $E\mu R$  *Igk* alleles allowed the direct comparison of the two alleles in the same reaction (Fig. 4 A). Consistent with the notion that rearrangement of the  $E\mu R$  allele is activated in pro-B cells, the levels of  $V_{\kappa}J_{\kappa 1-5}$  rearrangement of the  $E\mu R$  allele were considerably increased when compared with those of the WT allele in +/ $E\mu R$  pro-B cells (Fig. 4 E, left lane). However, in +/ $E\mu R$  pre-B cells,  $V_{\kappa}J_{\kappa 1-5}$  rearrangements of the WT allele were increased compared with those of the  $E\mu R$  allele (Fig. 4 E, middle lane), and, in splenic  $\kappa^+$  B cells,  $V_{\kappa}J_{\kappa 1-5}$  rearrangements of the WT allele appeared to surpass those of the  $E\mu R$  allele (Fig. 4 E, right lane). This trend of increasing contribution of the WT allele and decreasing contribution of the  $E\mu R$  allele at later stages of development suggests that the overall

rearrangement of the  $E\mu R$  allele is less efficient than WT in pre-B cells and beyond.

### $E\mu R$ does not support *Igk* rearrangement in pre-B cells

Productive rearrangement of the *Igk* allele in  $E\mu R$  pro-B cells would lead to IgM surface expression and direct transition to the immature B cell stage, whereas  $E\mu R$  pro-B cells with nonproductive *Igk* rearrangements would differentiate into pre-B cells. If this hypothesis is true, the rearrangements detected in  $E\mu R$  pre-B cells could be nonproductive ones that originated at the pro-B cell stage. To identify the origin of the  $E\mu R$  rearrangements detected in pre-B cells, we took advantage of the finding that terminal deoxynucleotidyl transferase is expressed exclusively in pro-B cells and mediates the nontemplated insertion of nucleotides, called N nucleotides, into the junctions of V, D, and J gene segments (47, 48). Because terminal deoxynucleotidyl transferase is not expressed in pre-B cells, N-nucleotide insertions rarely occur in  $V_{\kappa}J_{\kappa}$  junctions of WT B cells and can be regarded as a hallmark of V(D)J rearrangements that occurred at the pro-B cell stage. We amplified and sequenced  $V_{\kappa}J_{\kappa 1}$  junctions from  $E\mu R$  pro-, pre-, and mature B cells as well as from WT pre-B cells (Fig. 5 A). This analysis showed that 78% of  $V_{\kappa}J_{\kappa 1}$  junctions in  $E\mu R$  pro-B cells contained N nucleotides. Similarly, 67% of  $V_{\kappa}J_{\kappa 1}$  junctions in  $E\mu R$  pre-B cells contained N nucleotides in contrast to the 10% of  $V_{\kappa}J_{\kappa 1}$  junctions in WT pre-B cells that contained N nucleotides. In addition,  $V_{\kappa}J_{\kappa 1}$  rearrangements observed in  $E\mu R$  pre-B cells were



**Figure 5. Analysis of  $V_{\kappa}J_{\kappa}$  junctions and RS recombination in WT and  $E\mu R$  B cells.** (A) Percentages of total  $V_{\kappa}J_{\kappa 1}$  junctions with N-nucleotide insertions in sorted pro-, pre-, and mature B cells of WT and  $E\mu R$  mice. The number of sequences analyzed for  $E\mu R$  pro-,  $E\mu R$  pre-,  $E\mu R$  spleen  $\kappa^+$ , and WT pre-B cells are 22, 24, 43, and 12, respectively. P values for the comparison of N nucleotides in  $E\mu R$  pre-B cell populations to that of WT pre-B cells are shown (two-tailed paired Student's *t* test). (B) Detection of pre-B cell rearrangements by real-time LM-PCR of  $J_{\kappa 1}$  SE breaks in WT and  $E\mu R$  pre-B cells. Samples were normalized to the  $\beta$ -actin locus. SEs detected in  $E\mu R$  pre-B cells are shown relative to WT. Error

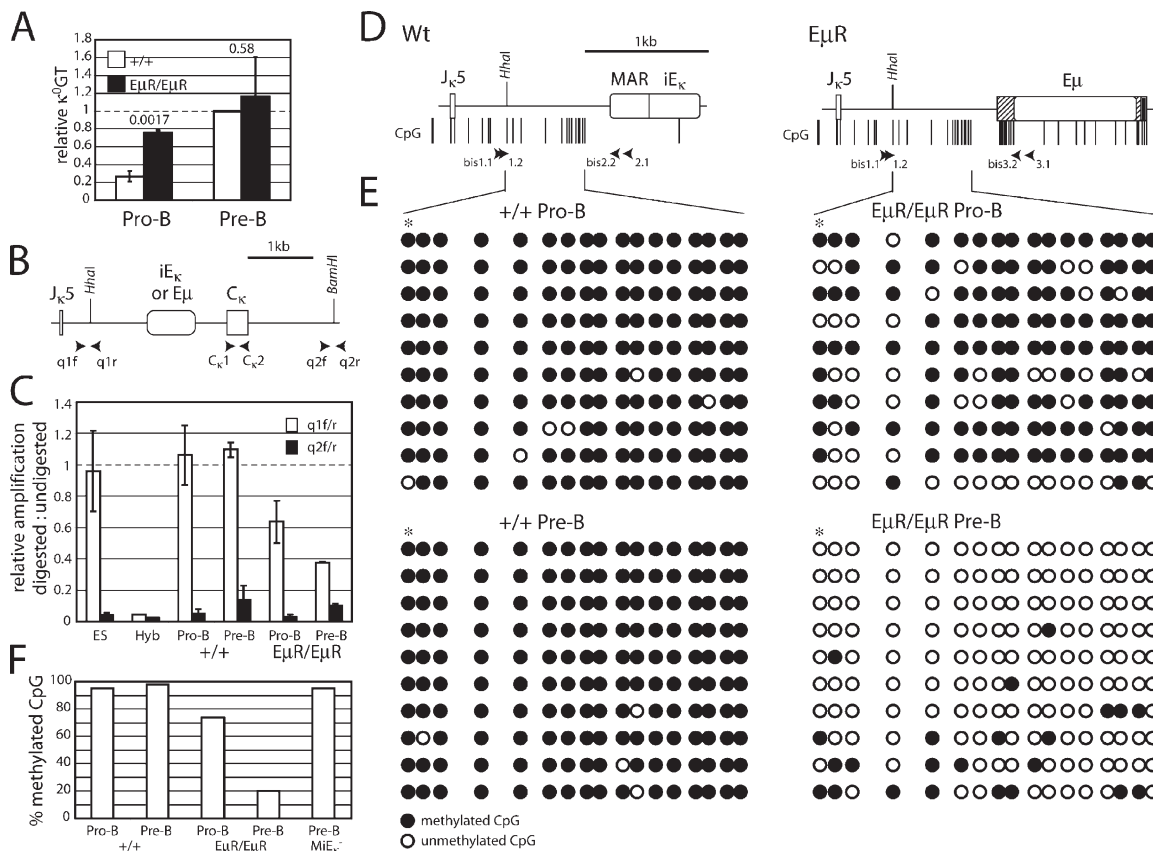
bar represents SD. (C) Proportions of several  $V_{\kappa}$  family members used in the  $V_{\kappa}J_{\kappa 1}$  rearrangements in the pro-, pre-, and splenic  $\kappa^+$  B cells of  $E\mu R$  mice as well as WT pre-B cells. The four most commonly used  $V_{\kappa}$  families are shown. (D and E) Analysis of RS rearrangement in WT and  $E\mu R$  pro- and pre-B cells. (D) Diagram of PCR strategy. Only alleles that have undergone rearrangement between the iRS and RS sequences will amplify. (E) Genomic DNA was serially diluted fivefold and amplified with the iRS and RS2 primers. The relative amount of genomic DNA in the most concentrated samples of the PCR reaction was determined by quantitative real-time PCR analysis of the  $\beta$ -actin gene and is shown at the bottom.

almost entirely nonproductive (21 of 24 sequences out of frame), suggesting that these rearrangements represent non-productive rearrangements carried over from the pro-B cell stage. In further support of this notion, >80% of  $V_{\kappa}J_{\kappa}5$  junctions (14 of 17 sequences) contain N nucleotides and are mostly out of frame in  $E_{\mu}R$  pre-B cells (unpublished data).

To further test whether the  $E_{\mu}R$  allele rearranges inefficiently in pre-B cells, we used a quantitative real-time ligation-mediated (LM) PCR assay to determine the level of the signal end (SE) double-strand breaks, a hallmark of RAG-induced cleavage, at the  $J_{\kappa}1$  gene segment in WT and  $E_{\mu}R$  pre-B cells as described previously (49). Compared with that in WT pre-B cells, the level of  $J_{\kappa}1$  SE breaks was reduced by

approximately fourfold in  $E_{\mu}R$  pre-B cells (Fig. 5 B). Considering that the level of unrearranged *Igk* alleles in  $E_{\mu}R$  pre-B cells was 60% of that in WT pre-B cells (Fig. 4 D), this suggested that the rearrangement frequency of the  $E_{\mu}R$  allele was only ~40% of that of the WT *Igk* allele in pre-B cells. Because a similar defect in *Igk* rearrangement was observed in  $MiE_{\kappa}$ -deleted mice, these findings indicate that  $E_{\mu}$  cannot functionally replace  $MiE_{\kappa}$  to support V(D)J rearrangement in pre-B cells.

Despite the high frequency of N-nucleotide insertions in  $V_{\kappa}J_{\kappa}1$  in  $E_{\mu}R$  pro- and pre-B cells, only 37% of  $V_{\kappa}J_{\kappa}1$  rearrangements from splenic  $\kappa^{+}$  B cells contained N nucleotides (Fig. 5 A), suggesting that pro-B cell-derived rearrangements



**Figure 6. Analysis of accessibility of the  $J_{\kappa}C_{\kappa}$  region in WT and  $E_{\mu}R$  pro- and pre-B cells.** (A) Relative  $\kappa^0GT$  expression in sorted pro- (left) and pre-B cells (right) of WT and  $E_{\mu}R$  mice.  $\kappa^0GT$  mRNA levels were normalized to the levels of  $\beta$ -actin mRNA. The  $\kappa^0GT$  levels of all samples are relative to those in WT pre-B cells. P values, which are shown in each graph, were generated using the two-tailed paired Student's *t* test (error bars represent SD; *n* = 3). (B) MSRE-QPCR strategy. Restriction sites and primer annealing locations are shown. (C) MSRE-QPCR analysis of genomic DNA derived from pro- and pre-B cells of WT and  $E_{\mu}R$  mice. Genomic DNA derived from ES cells and a hybridoma line (Hyb) was used as negative and positive controls. The ratio of the amplified products from the sample digested with *HhaI* versus those from the undigested sample is shown. The DNA amount used for PCR was normalized by the amplification of  $C_{\kappa}$  (primers  $C_{\kappa}1$  and  $C_{\kappa}2$ ). (D) CpG map in the  $J_{\kappa}C_{\kappa}$  intron.

Locations of the CpG dinucleotides are indicated by vertical lines below the map. Primers used to amplify bisulfite-treated DNA are indicated by arrowheads. (E) Methylation status of the 17 CpG dinucleotides within the  $J_{\kappa}C_{\kappa}$  intron in the pro- and pre-B cells of WT and  $E_{\mu}R$  mice. Each row of circles represents the methylation status of a single PCR product (one allele). 10 representative sequences from each sample are shown. The first circle (denoted by an asterisk) corresponds to the CpG site within the *HhaI* restriction site that was analyzed by MSRE-QPCR. (F) Percentage of the methylated CpG sites analyzed by bisulfite genomic sequencing. Percentages were calculated as the total number of methylated CpG sites divided by the total number of CpG sites analyzed. Bisulfite analysis of  $MiE_{\kappa}$ -deleted loci is shown in Fig. S3 (available at <http://www.jem.org/cgi/content/full/jem.20052310/DC1>).

may be negatively selected during development and, additionally, that some rearrangement can occur beyond the pro-B and pre-B cell stages in E $\mu$ R B cells. Recent studies suggest that 25–50% of mature B cells may have undergone receptor editing (50–52), a process by which recombination in light chain genes is reactivated to remove autoreactive antibodies (53–55). Therefore, it is likely that E $\mu$ R B cells also undergo robust receptor editing. We analyzed V $\kappa$  usage in B cells at various developmental stages in E $\mu$ R mice and WT mice and found that V $\kappa$ 2 family members were used frequently in V $\kappa$ J $\kappa$ 1 rearrangements in E $\mu$ R pro- and pre-B cells (50 and 37%, respectively) but less frequently in splenic B cells (12%). This is consistent with a change in the B cell repertoire between BM and peripheral B cells (Fig. 5 C).

Recombining sequence (RS) rearrangement between an RSS within the J $\kappa$ C $\kappa$  intron (iRS) and the RS sequence 21 kb downstream of C $\kappa$  is a primary mechanism for the receptor editing of *Igk* (56). To directly test whether E $\mu$ R B cells actively undergo receptor editing of *Igk*, we analyzed the frequency of RS recombination in E $\mu$ R and WT pro- and pre-B cells using a semiquantitative PCR assay as previously described (Fig. 5 D; reference 56). Although little RS rearrangement was detected in WT and E $\mu$ R pro-B cells, RS recombination was increased in E $\mu$ R pre-B cells when compared with that in WT pre-B cells, which is consistent with the findings that the J $\kappa$  region is accessible in E $\mu$ R pre-B cells (Fig. 5 E).

### The J $\kappa$ region becomes accessible in E $\mu$ R pro-B cells and remains so in pre-B cells

To understand the mechanisms by which E $\mu$ R activates *Igk* rearrangement at the pro-B cell stage, we analyzed  $\kappa$  germline transcription in pro- and pre-B cells sorted from WT and E $\mu$ R BM. A real-time quantitative PCR strategy was designed to determine the amount of GT initiating from promoters upstream of the J $\kappa$  gene segments ( $\kappa^0$ GT) using primers immediately upstream of J $\kappa$ 1 and within the C $\kappa$  exon. mRNA levels of  $\beta$ -actin were also analyzed by quantitative PCR and used as the control for the input RNA amount. Consistent with increased accessibility of *Igk* loci in E $\mu$ R pro-B cells, our analysis indicated an approximate threefold increase in the amount of  $\kappa^0$  transcripts in E $\mu$ R pro-B cells when compared with WT pro-B cells (Fig. 6 A). The amount of  $\kappa^0$  transcript in E $\mu$ R pre-B cells was similar to that in WT pre-B cells, suggesting that the *Igk* locus remains in an open configuration at the J $\kappa$  region in E $\mu$ R pre-B cells (Fig. 6 A).

Chromatin remodeling, including DNA demethylation, modulates the accessibility of Ig gene loci to the V(D)J recombinase (31, 57). Therefore, we compared the extent of DNA methylation at the *Igk* locus within the region spanning the J $\kappa$  and C $\kappa$  exons (J $\kappa$ C $\kappa$  intron) in E $\mu$ R and WT pro- and pre-B cells. The methylation status at an HhaI site residing within the J $\kappa$ C $\kappa$  intron just downstream of the J $\kappa$ 5 gene segment has been frequently analyzed as an indicator of the methylation status of the J $\kappa$ C $\kappa$  region (Fig. 6 B; references 32, 37, 58). We designed a quantitative real-time PCR assay to

detect the amount of DNA that was not cut at the HhaI site (methylated) using primers that flank that site (q1f and q1r) and compared it with undigested controls (Fig. 6 B). Consistent with previous findings, our analysis indicated little demethylation in the pro- or pre-B cells of WT mice (Fig. 6 C; reference 32). In contrast, J $\kappa$ C $\kappa$  demethylation was substantially increased in the pro-B cells of E $\mu$ R mice (Fig. 6 C). More strikingly, the majority of *Igk* loci appeared to be demethylated in the E $\mu$ R pre-B cells (Fig. 6 C). Therefore, this assay demonstrates that E $\mu$  has the ability to induce demethylation of the J $\kappa$ C $\kappa$  region in pro- and pre-B cells.

To confirm that the notable differences in methylation observed at the HhaI site are representative of the entire J $\kappa$ C $\kappa$  region, we analyzed the methylation status of adjacent CpG sites using bisulfite genomic sequencing (59). Consistent with the methylation-sensitive restriction enzyme real-time quantitative PCR (MSRE-QPCR) assay, in WT pro- and pre-B cells, *Igk* loci were hypermethylated at nearly all CpG sites examined (Fig. 6, B and E). In E $\mu$ R pro-B cells, *Igk* loci were partially demethylated with a mean of 25% (Fig. 6, E and F). This pattern is distinct from the monoallelic demethylation pattern of *Igk* loci observed in WT splenic B cells, where normally one allele is fully methylated and the other allele is fully demethylated (23, 32). In E $\mu$ R pre-B cells, the J $\kappa$ C $\kappa$  region was highly demethylated with a mean of 14.3 of 17 sites (86%) unmethylated per sequence (Fig. 6, E and F).

We have previously observed the hypomethylation of *Igk* loci in mature splenic MiE $\kappa$ <sup>-/-</sup> B cells, suggesting the possibility that the hypomethylation observed in E $\mu$ R loci is caused by the absence of MiE $\kappa$  (23). However, similar to the WT allele, the MiE $\kappa$ -deleted *Igk* locus is mostly methylated in pre-B cells (Fig. S3, available at <http://www.jem.org/cgi/content/full/jem.20052310/DC1>). Thus, the hypomethylation of the E $\mu$ R locus is caused by the presence of E $\mu$ , not the absence of MiE $\kappa$ . In conclusion, our findings indicate that E $\mu$  can promote robust chromatin remodeling of the J $\kappa$ C $\kappa$  region in both pro- and pre-B cells.

### The V $\kappa$ regions are accessible in E $\mu$ R pro-B cells, but not in pre-B cells

V(D)J recombination requires that both the V region and (D)J region are accessible to the recombination machinery. Because V $\kappa$  germline transcription (V $\kappa$ GT) predicts their accessibility to V(D)J recombinase (1), we examined germline transcription of five V $\kappa$  gene segments that spread throughout the locus in E $\mu$ R pro- and pre-B cells (Fig. 7 A). The amount of V $\kappa$ GT was quantitated by real-time PCR using primers annealing to the transcribed regions downstream of the V $\kappa$  RSS, which are deleted upon rearrangement and, thus, only amplify transcription from unrearranged V $\kappa$  promoters (Fig. 7 B). In pooled E $\mu$ R pro-B cells, the V $\kappa$  germline transcription of three of five V $\kappa$  genes was increased in E $\mu$ R pro-B cells when compared with WT pro-B cells (Fig. 7 C). Thus, consistent with the increased rearrangement of the E $\mu$ R allele in pro-B cells, E $\mu$ R appears to increase the accessibility of V $\kappa$  genes in pro-B cells.

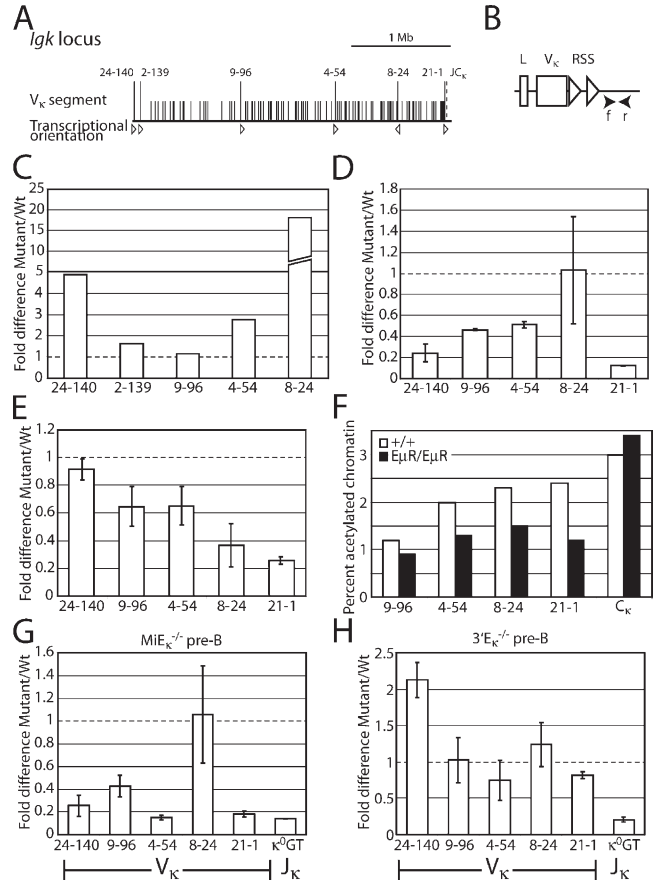
To understand the basis for the inefficient *Igk* rearrangement observed in E $\mu$ R pre-B cells, we also analyzed germline transcription of five V $\kappa$  segments in E $\mu$ R pre-B cells. Because the percentage of V $\kappa$  in germline configuration is likely to be different between WT and E $\mu$ R pre-B cells, the ratio of the amount of each germline V $\kappa$  in E $\mu$ R pre-B cells versus WT pre-B cells was analyzed by quantitative real-time PCR using primers that are specific to each unrearranged V $\kappa$  (Fig. 7 E). By normalizing the levels of V $\kappa$ GT to the amount of germline V $\kappa$ , germline transcription of four of the five V $\kappa$  genes examined was markedly reduced in E $\mu$ R pre-B cells when compared with that in WT pre-B cells, suggesting a reduced V $\kappa$  accessibility in E $\mu$ R pre-B cells (Fig. 7 D).

Histone acetylation is another hallmark for accessibility to the V(D)J recombinase (38). Therefore, we also examined histone acetylation of V $\kappa$  segments in pooled WT and E $\mu$ R pre-B cells by chromatin immunoprecipitation (ChIP) assays using an antibody against acetylated histone H3. To specifically detect only germline V $\kappa$  gene segments, we used the same primer sets that were used to analyze V $\kappa$ GT to determine the amount of germline V $\kappa$  in both immunoprecipitated chromatin and total input chromatin. In pooled pre-B cells sorted from five E $\mu$ R and five age-matched WT littermates, histone acetylation of the analyzed V $\kappa$  gene segments was generally reduced (Fig. 7 F). As a control, we also examined acetylation at the C $\kappa$  exon and found equivalent levels of acetylation between E $\mu$ R and WT pre-B cells, which is consistent with the observation that the J $\kappa$ C $\kappa$  region remains open in pre-B cells (Fig. 7 F). Together with the reduction in V $\kappa$ GT, these data indicate that the inefficient *Igk* rearrangement observed in E $\mu$ R pre-B cells is caused by the reduced accessibility of V $\kappa$  regions to the V(D)J recombinase.

The reduction in V $\kappa$  accessibility observed in E $\mu$ R pre-B cells could be caused by the presence of E $\mu$  and/or the absence of MiE $\kappa$ . To distinguish between these possibilities, we analyzed V $\kappa$  germline transcription in pre-B cells sorted from MiE $\kappa$ <sup>-/-</sup> mice. Germline transcription of most V $\kappa$  genes analyzed was considerably reduced in MiE $\kappa$ <sup>-/-</sup> pre-B cells compared with that in WT pre-B cells, indicating that MiE $\kappa$  is required for the accessibility of V $\kappa$  in pre-B cells (Fig. 7 G). In contrast to that in E $\mu$ R pre-B cells,  $\kappa^0$ GT was dramatically reduced in MiE $\kappa$ <sup>-/-</sup> pre-B cells (Fig. 7 G). V $\kappa$ GT was normal in 3'E $\kappa$ <sup>-/-</sup> pre-B cells, but, consistent with previous studies (22, 23),  $\kappa^0$ GT was reduced (Fig. 7 H). Therefore, MiE $\kappa$  plays an important role in promoting the accessibility of both V $\kappa$  and J $\kappa$  regions in pre-B cells, and E $\mu$  can compensate for MiE $\kappa$  in opening up the J $\kappa$  region but not the V $\kappa$  region in E $\mu$ R pre-B cells.

**DISCUSSION**

It has been well established that V(D)J recombination of the Ig heavy and light chain genes occur in a sequential manner (1). In this context, the rearrangement of *IgH* chain genes occurs exclusively in pro-B cells, whereas the rearrangement of *IgL* chain genes occurs primarily in pre-B cells. Our findings indicate that E $\mu$ R replacement greatly increases *Igk*



**Figure 7. Analysis of V $\kappa$  gene accessibility in *Igk* mutant mice.** (A) Map of *Igk* locus. Locations of V $\kappa$  genes are represented by vertical lines. The six V $\kappa$  genes analyzed are labeled above and are represented with longer lines. The transcriptional orientation of each of the six V $\kappa$  genes is indicated by arrowheads below. The J $\kappa$ C $\kappa$  region is represented by a broken line. (B) Real-time PCR strategy to quantitate the amount of V $\kappa$  germline transcription (V $\kappa$ GT). A generic map of unrearranged V $\kappa$  gene segment is shown with leader (L) and V $\kappa$  exons (boxes) and the RSS sequence (triangles). Forward (f) and reverse (r) primers are indicated by arrowheads. (C) V $\kappa$  germline transcription (V $\kappa$ GT) in pooled WT and E $\mu$ R pro-B cells. The amount of GTs of five V $\kappa$  genes was analyzed by quantitative real-time PCR and normalized to CD19 mRNA levels. The thick horizontal line at the relative V $\kappa$ GT value of five indicates a change in scale. (D) V $\kappa$ GT in WT and E $\mu$ R pre-B cells. All V $\kappa$ GT levels were normalized to the levels of CD19 mRNA and the relative amount of unrearranged V $\kappa$  alleles in E $\mu$ R pre-B cells versus that in WT pre-B cells, as shown in E. (C and D) The y axis represents the ratio of V $\kappa$ GT in E $\mu$ R pro-/pre-B cells versus that in WT pro-/pre-B cells. Error bars represent SD. (E) V $\kappa$ GT in WT and E $\mu$ R pre-B cells. All V $\kappa$ GT levels were normalized to the levels of CD19 mRNA and the relative amount of unrearranged V $\kappa$  alleles in E $\mu$ R pre-B cells versus that in WT pre-B cells, as shown in E. (C and D) The y axis represents the ratio of V $\kappa$ GT in E $\mu$ R pro-/pre-B cells versus that in WT pro-/pre-B cells. Error bars represent SD. (F) Histone acetylation of V $\kappa$  in pools of WT and E $\mu$ R pre-B cells analyzed by ChIP. The V regions analyzed are labeled on the x axis, and the percentage of chromatin immunoprecipitated from the total input chromatin is shown on the y axis. The primers used for the V $\kappa$  genes are the same as those used for the V $\kappa$ GT analysis, and the C $\kappa$  primers are the same as used in Fig. 5 B. (G and H) Germline transcription of V $\kappa$  and J $\kappa$  regions in MiE $\kappa$ <sup>-/-</sup> (G) and 3'E $\kappa$ <sup>-/-</sup> (H) pre-B cells. The ratio of the GT levels in mutant pre-B cells versus those in WT pre-B cells is shown on the y axis.



rearrangement in pro-B cells. In addition, the germline transcription of both  $V_{\kappa}$  and  $J_{\kappa}$  regions as well as DNA demethylation at the  $J_{\kappa}$  region are substantially increased in  $E\mu R$  pro-B cells, indicating that  $E\mu$  is sufficient to promote the accessibility of these loci to  $V(D)J$  recombinase and activate  $V(D)J$  recombination in pro-B cells.

To maintain the sequential rearrangement of *IgH* and *IgL* chain genes, the rearrangement of *IgH* loci occurs in pro-B cells but not in pre-B cells. Analysis of B cell development and *Igk* rearrangement suggests that *Igk* rearrangement is not efficient in  $E\mu R$  pre-B cells. In further support of this notion, the percentage of  $V_{\kappa}J_{\kappa}$  junctions containing N nucleotides is similar in  $E\mu R$  pro-B and pre-B cells, indicating that the majority of the *Igk* rearrangements detected in  $E\mu R$  pre-B cells have occurred in pro-B cells. In addition, the level of RAG-induced SE breaks is markedly reduced in  $E\mu R$  pre-B cells. Therefore,  $E\mu$  promotes  $V(D)J$  recombination in pro-B cells but cannot support efficient  $V(D)J$  rearrangement in pre-B cells, further implicating its importance in maintaining the sequential rearrangement of *IgH* and *IgL* chain genes.

Efficient  $V(D)J$  recombination requires that both V and J regions are accessible to the  $V(D)J$  recombinase. Because the germline transcription and histone acetylation through  $J_{\kappa}$  is normal in  $E\mu R$  pre-B cells, the inefficient rearrangement of the  $E\mu R$  allele in pre-B cells is not caused by the inaccessibility of the  $J_{\kappa}$  region to the  $V(D)J$  recombinase. In contrast, germline transcription and histone acetylation of unrearranged  $V_{\kappa}$  gene segments are noticeably reduced in  $E\mu R$  pre-B cells, indicating that accessibility of the  $V_{\kappa}$  region is reduced in  $E\mu R$  pre-B cells. This scenario is remarkably similar to the *IgH* locus in normal pre-B cells, where the  $V_H$  region becomes inaccessible but the  $DJ_H$  region remains accessible (35). As germline transcription of  $V_{\kappa}$  and  $J_{\kappa}$  is considerably reduced in  $MiE_{\kappa}^{-/-}$  pre-B cells, the reduced accessibility of  $V_{\kappa}$  in  $E\mu R$  pre-B cells is caused by the lack of  $MiE_{\kappa}$ . Therefore,  $E\mu$  and  $MiE_{\kappa}$  maintain the sequential rearrangement of the Ig loci by playing developmental stage-specific roles in promoting the chromatin remodeling at V regions.

Because Ig enhancers are essentially clusters of protein-binding sites, the function of an enhancer is dictated by the array of DNA-binding factors it recruits. A comparison of the known functional motifs within  $E\mu$  and  $MiE_{\kappa}$  shows that  $\mu A$  and  $\mu B$  sites are only present in  $E\mu$ . The binding of PU.1 and ETS family proteins to  $\mu A$  and  $\mu B$  sites promotes chromatin accessibility to endonucleases (60). In addition, although two E2A-binding E boxes, which are important for activating *Igk* rearrangement, are present in  $MiE_{\kappa}$  (40), three E2A-binding E boxes are present in  $E\mu$  (61). The unique combination of functional motifs within  $E\mu$  might account for its activities in activating  $V(D)J$  recombination in pro-B cells.

It remains unclear why  $E\mu$  cannot compensate for  $MiE_{\kappa}$  function in promoting the accessibility of  $V_{\kappa}$  regions in pre-B cells. Most of the known protein-binding sites within  $MiE_{\kappa}$  are also found in  $E\mu$ . One exception is the NF- $\kappa B$ -binding site. However, deletion of this site has no considerable impact on *Igk* rearrangement (40). Both  $MiE_{\kappa}$  and  $E\mu$  con-

tain MARs that could bind to the nuclear matrix and extend the range of the action of its associated enhancers (62–65). It is possible that the MARs of  $E\mu$  and  $MiE_{\kappa}$  function differently in promoting  $V_{\kappa}$  accessibility in pre-B cells. Although neither the two MARs of  $E\mu$  nor the MAR of  $MiE_{\kappa}$  is required for rearrangement (66, 67), increased premature *Igk* rearrangement in pro-B cells has been detected in mice with a deletion of MAR of  $MiE_{\kappa}$  (67, 68). It is possible that certain unidentified elements within  $MiE_{\kappa}$  are required for promoting the accessibility of  $V_{\kappa}$  in pre-B cells. Alternatively, some elements within  $E\mu$ , such as  $\mu NR$ , might dominantly suppress its activity in promoting accessibility in V regions in pre-B cells (69).

Recent studies indicate that during recombination, Ig loci migrate to the nuclear center (70), where they contract (71) via DNA-looping (72, 73) mechanisms that bring distal V segments in close proximity to the enhancers (74). The ability of  $E\mu$  and  $MiE_{\kappa}$  to regulate V-region accessibility as far as 3 Mb away suggests that these enhancers may also play a role in regulating higher order chromatin modifications. Further analysis of the chromatin remodeling in  $E\mu R$  and  $MiE_{\kappa}^{-/-}$  precursor B cells will help to define the functions of intronic enhancers in regulating such processes. In summary, the *IgH* and *Igk* intronic enhancers activate the sequential rearrangement of Ig loci by inducing stage-specific chromatin remodeling events in regions proximal to the enhancers and coordinate with V promoters to induce accessibility at distal V regions.

## MATERIALS AND METHODS

**Generation of  $E\mu R$  ES cells and knockin mice.**  $E\mu$  was cloned into pBluescript and inserted into a targeting construct that was used previously to knockout the *Igk* intronic enhancer (21).  $E\mu$  was inserted into the *Sall* site upstream of the floxed neomycin resistance gene under control of the PGK promoter (*f-PGK-neo<sup>r</sup>*), the same insertion site previously used to reintroduce mutant  $MiE_{\kappa}$  into the targeting construct (40). The targeting construct was linearized with *PvuI* and was electroporated into ES cells as described previously (75). Transfected ES cells were cultured with 300  $\mu\text{g/ml}$  G418 and 1  $\mu\text{M}$  gancyclovir to select for integration by homologous recombination. Positive clones were first screened by PCR and then by Southern blotting, digesting with *EcoRI* and probing with probe A as previously described (40). The WT locus produces a 15.1-kb fragment, whereas the targeted locus produces a 12.2-kb fragment. The floxed *PGK-neo<sup>r</sup>* was removed through *Cre/loxP*-mediated deletion as described previously (21) and was screened by PCR and Southern blotting under the same conditions. The final configuration, called  $E\mu R$ , produces an 11.0-kb fragment. Positive clones were then subcloned and rescreened to ensure purity.  $E\mu R$  ES cells were injected into mouse blastocysts to produce chimeric mice, which were bred and screened for germline transmission of the  $E\mu R$  allele.

**Cell purification and flow cytometry.** BM and spleen cells were harvested in FACS buffer (3% FCS in PBS) and purified into single-cell suspensions. For total white blood cell counts, splenocytes were counted in 2% glacial acetic acid. For purification and flow cytometry, red blood cells were removed by treatment with red blood cell lysis buffer (0.15 M  $\text{NH}_4\text{Cl}$ , 1 mM  $\text{KHCO}_3$ , and 0.1 mM  $\text{Na}_2\text{EDTA}$ , pH 7.0) for 5 min at room temperature. 1 million cells were pretreated with 1  $\mu\text{g}$  FcBlock (BD Biosciences) and stained in 100- $\mu\text{l}$  reactions on ice for 15 min. If necessary, cells were washed and stained with streptavidin-coated dyes under the same conditions. Cells were resuspended in 0.5  $\mu\text{g/ml}$  propidium iodide in FACS buffer to stain for dead cells. For spleen cell stains, live cells within the lymphocyte gate were analyzed. Additionally, to exclude clumped cells, only small lymphocytes were analyzed.

For BM stains, live lymphocytes and granulocytes were analyzed. Antibodies used are as follows: anti-B220-PE (BD Biosciences), anti-IgM-FITC (BD Biosciences), anti-CD43-biotin (BD Biosciences), anti-mouse Igk-FITC (BD Biosciences), anti-mouse Igλ-biotin (BD Biosciences), anti-CD3-FITC (BD Biosciences), streptavidin-APC (eBioscience), and streptavidin-PECy5 (eBioscience). To obtain purified pro- and pre-B cells, BM was stained with B220-PE, IgM-FITC, and CD43-biotin and was washed and restained with streptavidin-PECy5. Pro-B cells (B220<sup>+</sup>, IgM<sup>-</sup>, and CD43<sup>+</sup>) and pre-B cells (B220<sup>+</sup>, IgM<sup>-</sup>, and CD43<sup>-</sup>) were sorted using a FACSVantage Cell Sorter (Becton Dickinson). κ<sup>+</sup> spleen cells were sorted by negative selection using the MACS system (Miltenyi Biotec), anti-Igλ-biotin antibodies, and anti-CD43 and anti-biotin microbeads according to the manufacturer's instructions and as previously described (40). The purity of sorted B cells was confirmed by FACS analysis. All purifications used were >95% B220<sup>+</sup>.

**Primer sequences.** Sequences of all primers used are listed in the supplemental material (available at <http://www.jem.org/cgi/content/full/jem.20052310/DC1>).

**Real-time quantitative RT-PCR analysis.** RNA from sorted pro-, pre-, and κ<sup>+</sup> splenic B cells was purified using the RNeasy Purification Kit (QIAGEN) as described previously (40). RNA was converted to cDNA using the Superscript first strand cDNA synthesis kit (Invitrogen) as described previously (40). cDNA was amplified using the 2× SYBR Green Master Mix (Applied Biosystems) in a sequence detection system (Prism 7000; ABI Biosystems). PCR results were analyzed using Prism 7000 SDS software (ABI Biosystems) and relative transcription calculated using the ΔC<sub>t</sub> method according to the manufacturer's instructions.

**Rearrangement PCR assay.** DNA was purified from sorted B cell populations as described previously (23). Rearranged *Igk* alleles were amplified using the degenerate V<sub>κ</sub> primer (V<sub>κ</sub>D) and either primer K6 or primer MAR35 under PCR conditions that were previously described (23, 40). PCR products were run on an agarose gel, transferred to a membrane, and probed as previously described (23). Heavy chain rearrangement was amplified by PCR and analyzed by Southern blotting as previously described (40). RS rearrangement was analyzed with a PCR assay with primers iRS and a primer downstream of the RS RSS (primer R2) as previously described (56). The intensity of each band was calculated using a phosphorimager (Storm; GE Healthcare) as previously described (23).

**Cloning and sequencing of V<sub>κ</sub>J<sub>κ</sub>1 rearrangements.** Rearranged *Igk* alleles were amplified using KOD Hotstart Polymerase (Calbiochem) according to the manufacturer's instructions. A degenerate V<sub>κ</sub> primer with a BamHI restriction sequence inserted (V<sub>κ</sub>D.bam) and a primer that anneals between J<sub>κ</sub>1 and J<sub>κ</sub>2 with an EcoRI restriction sequence added (J<sub>κ</sub>1.eco) were used. PCR products were purified using the Wizard SV PCR and Gel Clean-Up System (Promega) according to the manufacturer's instructions, digested with BamHI and EcoRI, and cloned into pBluescript. Clones were sequenced by Eton Biosciences using the T3 primer. Sequences were analyzed by IgBLAST software, which is available from the National Center for Biotechnology Information's website (<http://www.ncbi.nlm.nih.gov/igblast/>) to determine the V<sub>κ</sub> gene segment used and to identify junctional insertions and deletions. N-nucleotide insertions were determined using previously described methods (48). In brief, insertions with sequences complementary to V<sub>κ</sub> or J<sub>κ</sub> ends were classified as P nucleotides. Insertions that could not be classified as P nucleotides were considered N nucleotides. For example, in the hypothetical sequence ACCGGCTGG, where the underlined sequences correspond to V<sub>κ</sub> (left) and J<sub>κ</sub>1 (right), GGC would represent inserted nucleotides. Because the inserted GG nucleotides are complementary to the V<sub>κ</sub> end, they would be classified as P nucleotides. The inserted C nucleotide, which is not complementary to either V<sub>κ</sub> or J<sub>κ</sub> ends, would be classified as an N nucleotide.

**Detection of SE breaks by quantitative LM-PCR.** Genomic DNA was purified from sorted pre-B cell populations as described previously (49).

LM-PCR was performed essentially as described previously with all of the reagents provided by J. Curry of M. Schissel's laboratory (University of California, Berkeley, CA; reference 49). In brief, 500 ng of genomic DNA was used in each ligation reaction. Quantitative real-time PCR analysis of the levels of the ligation product of J<sub>κ</sub>1 SE break was performed exactly as described previously (49). The amount of input genomic DNA was determined by quantitative real-time PCR with primers specific for the β-actin promoter.

**MSRE-QPCR.** DNA from sorted B cells was digested in a 50-μl reaction volume with 20 U EcoRI (New England Biolabs, Inc.) for 1 h at 37°C. Subsequently, 23 μl were transferred to a separate tube, and 20 U of both BamHI and HhaI were added. The original EcoRI digest and the EcoRI, BamHI, and HhaI triple digests were incubated overnight at 37°C. Restriction enzymes were inactivated by heating at 65°C for 20 min. Approximately 0.5 μl of digest product were used per well for real-time quantitative PCR.

**Bisulfite genomic sequencing.** DNA from sorted B cells was converted with sodium bisulfite using the EZ DNA Methylation Kit (Zymo Research) according to the manufacturer's instructions. Converted DNA was amplified in two rounds of PCR. In the first round, bis1.1 and either bis2.1 or bis3.1 primers were used in the following touchdown PCR program: a 5-min 95°C initial incubation followed by 15 cycles of 94°C for 1 min, x°C for 1 min, and 72°C for 2 min and 30 s, where x is 64°C for the first cycle and lowers 1°C per cycle. This was followed by 15 cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 2 min and 30 s, and a final extension for 10 min at 72°C. 5–10% of the PCR product was used in a second round of PCR using primer bis1.2eco and either bis2.2bam or bis3.2bam and using KOD Hotstart Polymerase (Calbiochem) according to the manufacturer's protocol. The PCR program for the second round was 95°C for 2 min followed by 32 cycles of 94°C for 20 s, 55°C for 30 s, and 68°C for 2 min and 30 s. This was followed by a final extension for 10 min at 68°C. PCR products were purified using the Wizard SV Gel and PCR Clean-Up System (Promega), digested with BamHI and EcoRI, gel purified, and cloned into pBluescript. Sequencing was provided by the Cancer Center sequencing facility of the University of California, San Diego (UCSD).

**ChIP.** Sorted pre-B cells were pooled and fixed in 1% formaldehyde. Subsequent steps followed a previously described protocol (76) with some modifications. Fixed cells were lysed in L1 lysis buffer (50 mM Tris, pH 8.0, 2 mM EDTA, 0.1% NP-40, and 10% glycerol) supplemented with protease inhibitors. Nuclei were pelleted and resuspended in 300 μl L2 lysis buffer (50 mM Tris, pH 8.0, 0.1% SDS, and 5 mM EDTA) with protease inhibitors. Chromatin was fragmented by sonication, centrifuged, and diluted in dilution buffer (50 mM Tris, pH 8.0, 5 mM EDTA, 0.2 M NaCl, and 0.5% NP-40). After preclearing, immunoprecipitation was performed overnight at 4°C using 3 μl antiacetylated histone H3 antibody (Upstate Biotechnology). Immune complexes were collected with ssProtein A/G for 30 min and washed three times in washing buffer (20 mM Tris, pH 8.0, 0.1% SDS, 0.5 M NaCl, 2 mM EDTA, and 1% NP-40) and once in 0.5 M LiCl followed by three washes with TE buffer (10 mM Tris and 1 mM EDTA). Immune complexes were extracted three times with 100 μl of extraction buffer (TE buffer containing 2% SDS). DNA-protein cross-linking was reversed by heating at 65°C for 8 h. After proteinase K (100 μg for 2 h) digestion, DNA was extracted with phenol/chloroform and precipitated in ethanol. The UCSD Animal Subjects Committee approved all experiments that involved mice.

**Online supplemental material.** Fig. S1 shows the analysis of B cell populations in MiE<sub>κ</sub><sup>-/-</sup> BM and spleen by flow cytometry. Fig. S2 shows the analysis of B cell populations in EμR × hC<sub>κ</sub> BM and spleen. Fig. S3 shows the analysis of methylation in MiE<sub>κ</sub><sup>+/-</sup> pre-B cells. Supplemental material also provides a list of all primer sequences used. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20052310/DC1>.

We thank Drs. John Curry and Mark Schlissel for all reagents for the LM-PCR assay. We also thank Drs. David Baltimore, Stephen Hedrick, Cornelis Murre, David Nemazee, and Eugene Oltz for critical reading of this manuscript.

This work was supported by a National Institutes of Health grant (AI44838) to Y. Xu.

The authors have no conflicting financial interests.

Submitted: 17 November 2005

Accepted: 19 May 2006

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