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Title
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Permalink
https://escholarship.org/uc/item/0822g15w

Journal
The Journal of experimental medicine, 203(7)

ISSN
0022-1007

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Publication Date
2006-07-01

DOI
10.1084/jem.20052310

Peer reviewed
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 (MiEκ) with its heavy chain counterpart (Eμ) in mice. This replacement, denoted EμR, substantially increases the accessibility of both Vκ and Jκ loci to V(D)J recombinase in pro–B cells and induces Igκ rearrangement in these cells. However, EμR does not support Igκ rearrangement in pre–B cells. Similar to that in MiEκ−/− pre–B cells, the accessibility of Vκ segments to V(D)J recombinase is considerably reduced in EμR pre–B cells when compared with wild-type pre–B cells. Therefore, Eμ and MiEκ 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.

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. 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. 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 (Eμ) and matrix attachment region (MAR)/Igk intronic enhancer (MiEκ), respectively (8–12). Both loci also contain multiple other regulatory elements downstream or within their constant region exons. The Igk locus contains the 3′Eκ (13) and Ed (14) enhancers, and the IgH locus contains four enhancer elements in its 3′Eμ regulatory region (hs3a, hs1/2, hs3b, and hs4; references 15–17). The deletion of Eμ in mice severely decreases VH to DJH rearrangement (18–20). The deletion of either MiEκ or 3′Eκ 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 Jκ (24–27), as κ0 germline transcription (κ0GT [germline transcript]) appears to play a quantitative role in activating Igk rearrangement (28, 29). Germline transcription of Vκ gene segments (VκGT) may also play a role in regulating the accessibility of the Vκ 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...
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_{H1}–J_{H1} regions occurs during D_{H1} to J_{H1} rearrangement, and acetylation of V_{H1} histones occurs during V_{H1} to D_{H1} rearrangement (34). However, V_{H1} 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 occurs 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_{κ} 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 Eμ might be involved in this developmental stage-specific process. To test this hypothesis, we replaced the endogenous MiE_{κ} with Eμ in mice.

**RESULTS**

**Generation of EμR mice**

To replace the endogenous Igk intronic enhancer with the heavy chain intronic enhancer (Eμ), the 0.8-kb XbaI fragment spanning the entire Eμ element and flanking MAR sequences was inserted 5′ of a phosphoglycerol kinase (PGK)–Neo′ gene (f-PGK–Neo′). This enhancer replacement cassette was placed into a targeting vector that was used previously to delete Igk intronic enhancer (39, 40) and V_{κ} promoters (41). Homologous recombination was confirmed by Southern blotting with EcoRI digestion of genomic DNA and hybridization to probe A. First lane, WT ES cell DNA; second lane, heterozygous EμR; third lane, heterozygous EμR inserted. The size of the mutant EcoRI restriction fragment is shown. (D) Knockin allele with f-PGK–Neo′ removed by Cre/loxP-mediated deletion. (E) Southern blotting analysis of ES cell genomic DNA digested with EcoRI and probed with probe A. First lane, WT ES cell DNA; second lane, heterozygous EμR ES cell with PGK–Neo′ inserted; third lane, heterozygous EμR knockin with PGK–Neo′ deleted. Bands corresponding to the WT, EμR with PGK–Neo′ inserted, and knockin alleles are indicated on the right. B, BamHI; E, EcoRI; H, HindIII; X, XbaI.

**EμR mice exhibit moderate defects in κ^{+} B cell development**

To determine the effects of the EμR mutation on B cell development, BM and spleen cells derived from WT and EμR mice were analyzed by flow cytometry. In spleens from EμR mice, we found an ~50% decrease in the total number of B cells compared with WT controls (Fig. 2 C). The percentage of κ^{+} splenic B cells was reduced, lowering the κ/λ ratio from 20:1 in WT mice to 6:1 in EμR mice (Fig. 2, B and D). These data indicate an impairment in the development of κ^{+} 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 EμR splenic κ^{+} 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 (B_{220}^{+}CD43^{−}IgM^{−}) and a decrease in immature B cells (B_{220}^{+}IgM^{+}IgD^{lo}) was detected in EμR BM compared with those in WT controls (Fig. 3, A–C). Because the percentage of pre-B cells was similar between WT and EμR BM, these data indicate that the defect in EμR B cell development originates at the pre-B cell stage, where Igk

**Figure 1. Generation of EμR 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_{κ} with Eμ (striped rounded box) and floxed PGK–Neo′ (f-PGK–Neo′; striped box). (C) Targeted locus with f-PGK–Neo′ inserted. The size of the mutant EcoRI restriction fragment is shown. (D) Knockin allele with f-PGK–Neo′ removed by Cre/loxP-mediated deletion. (E) Southern blotting analysis of ES cell genomic DNA digested with EcoRI and probed with probe A. First lane, WT ES cell DNA; second lane, heterozygous EμR ES cell with PGK–Neo′ inserted; third lane, heterozygous EμR knockin with PGK–Neo′ deleted. Bands corresponding to the WT, EμR with PGK–Neo′ inserted, and knockin alleles are indicated on the right. B, BamHI; E, EcoRI; H, HindIII; X, XbaI.
normally rearranges (Fig. 3 D). This partial developmental block is similar to that observed in MiEκ−/− mice (Fig. S1, A, B, and E; available at http://www.jem.org/cgi/content/full/jem.20052310/DC1). However, MiEκ−/− mice have a much more severe decrease in the κ/λ ratio in splenic B cells than that observed in EμR mice (Fig. S1, B and F).

To directly compare the overall rearrangement efficiency of the EμR and WT allele, we bred EμR mice with knockin mice with their mouse κ constant region (mCκ) replaced with the human κ constant region (hCκ; Fig. S2 A, available at http://www.jem.org/cgi/content/full/jem.20052310/DC1). Analysis of +/hCκ and EμR/hCκ mice showed that they had similar populations of B cells in BM
In the spleen, +/-hCκ mice had approximately equal numbers of hCκ+ and mCκ+ B cells (Fig. S2, C and D). However, EuR/hCκ mice had a dramatic reduction in the percentage of mCκ+ B cells with a ratio of hCκ+/mCκ+ B cells of ~6:1 (Fig. S2, C and D). Therefore, the overall efficiency of the EuR allele appears to be lower than that of the WT allele.

Igk rearrangement is activated by EuR in pro-B cells

To examine whether EuR affects the timing of V(D)J recombination, we used a PCR assay to analyze Igk rearrangement in WT and EuR homozygous pro- and pre-B cells (Fig. 4 A). The degenerate Vκ primer (VκD), which binds to ~90% of Vκ gene segments, was used together with a primer downstream of Jκ5 (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 EuR allele is dramatically increased (~16-fold) in the pro-B cells of EuR mice (Fig. 4 B and C). At the pre-B cell stage, the levels of Igk rearrangement were similar between the WT and EuR mice (Fig. 4 B, right).

To estimate the level of rearrangement of EuR allele in pro-B cells, we designed a real-time PCR assay using primers that flanked the Jκ1 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 cells, we also used a real-time PCR assay using primers that flanked the Vκ and Jκ1 gene segments of the WT allele and EuR allele to analyze the level of unrearranged Igk allele in pro-B cells (Fig. 4 D, bottom). The primers 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 EuR/EuR pre-B cells by those in ES cells. Error bars represent SD.

Figure 4. Igk rearrangement in +/-, EuR/EuR, and +/-EuR mice.

(A) PCR strategy to detect Igk rearrangement. Configuration of the rearranged Igk locus in WT and EuR B lineage cells. Location of the PCR primers and probe and the size of the PCR product for a Vκ to Jκ1 rearrangement are shown. (B) Semiquantitative PCR analysis of Igk rearrangement using primers VκD and MAR35 in the pro- and pre-B cells of WT and EuR mice. Genomic DNA was serially diluted fourfold. Bands corresponding to the rearrangement of Vκ to each of the four Jκ gene segments are indicated on the left. To control for the amount of genomic DNA used for PCR analysis, rearrangement of the IgH locus (VDJκ) 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 amplified product of VκJκ rearrangement is normalized to the intensity of VDJκ rearrangement. (D) Percentage of unrearranged Igk alleles (κGL) in WT and EuR 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 EuR/EuR pre-B cells by those in ES cells. Error bars represent SD. (E) PCR amplification of rearranged Igk alleles in sorted heterozygous (+/-EuR) B lineage cells. Genomic DNA from sorted pro-, pre-, and mature B cells was amplified with the degenerate Vκ primer and primer K2. Bands corresponding to the rearrangements of Vκ to each of the four Jκ gene segments of the WT allele and EuR 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 \(\sim 90\) 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 \(\sim 50\%\) of WT \(Igk\) alleles were unrearranged in pre-B cells, whereas \(\sim 30\%\) 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 \(V_\kappa J_1-5\) 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, \(V_\kappa J_1-5\) 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, \(V_\kappa J_1-5\) 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 \(V_{\kappa k}\) 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 \(V_\kappa J_1\) junctions from \(E_{\mu R}\) pro-B, pre-B, and mature B cells as well as from WT pre-B cells (Fig. 5 A). This analysis showed that 78% of \(V_\kappa J_1\) junctions in \(E_{\mu R}\) pro-B cells contained N nucleotides. Similarly, 67% of \(V_\kappa J_1\) junctions in \(E_{\mu R}\) pre-B cells contained N nucleotides in contrast to the 10% of \(V_\kappa J_1\) junctions in WT pre-B cells that contained N nucleotides. In addition, \(V_\kappa J_1\) rearrangements observed in \(E_{\mu R}\) pre-B cells were

**Figure 5. Analysis of \(V_\kappa J_1\) junctions and RS recombination in WT and \(E_{\mu R}\) B cells.** (A) Percentages of total \(V_\kappa J_1\) 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-B, \(E_{\mu R}\) pre-B, \(E_{\mu R}\) 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 \(V_\kappa J_1\) 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 nonproductive rearrangements carried over from the pro–B cell stage. In further support of this notion, >80% of VκJκ5 junctions (14 of 17 sequences) contain N nucleotides and are mostly out of frame in EμR pre–B cells (unpublished data).

To further test whether the EμR 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 Jκ1 gene segment in WT and EμR pre–B cells as described previously (49). Compared with that in WT pre–B cells, the level of Jκ1 SE breaks was reduced by approximately fourfold in EμR pre–B cells (Fig. 5 B). Considering that the level of unrearranged Igk alleles in EμR pre–B cells was 60% of that in WT pre–B cells (Fig. 4 D), this suggested that the rearrangement frequency of the EμR 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 MIEκ-deleted mice, these findings indicate that Eμ cannot functionally replace MIEκ to support V(D)J rearrangement in pre–B cells.

Despite the high frequency of N-nucleotide insertions in VκJκ1 in EμR pro– and pre–B cells, only 37% of VκJκ1 rearrangements from splenic κ⁺ B cells contained N nucleotides (Fig. 5 A), suggesting that pro–B cell–derived rearrangements

Figure 6. Analysis of accessibility of the JCκ region in WT and EμR pro– and pre–B cells. (A) Relative κGT expression in sorted pro– (left) and pre–B cells (right) of WT and EμR mice. κGT mRNA levels were normalized to the levels of β-actin mRNA. The κGT 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 EμR 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 Cκ (primers Cκ1 and Cκ2). (D) CpG map in the JκCκ 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 JκCκ intron in the pro– and pre–B cells of WT and EμR 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 MIEκ-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 EμR 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 autoimmune antibodies (53–55). Therefore, it is likely that EμR B cells also undergo robust receptor editing. We analyzed Vκ usage in B cells at various developmental stages in EμR mice and WT mice and found that Vκ2 family members were used frequently in VκJκ1 rearrangements in EμR 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).

Recombinating sequence (RS) rearrangement between an RSS within the JκCκ intron (iRS) and the RS sequence 21 kb downstream of Cκ is a primary mechanism for the recombination of Igk (56). To directly test whether EμR B cells actively undergo receptor editing of Igk, we analyzed the frequency of RS recombination in EμR 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 EμR pro–B cells, RS recombination was increased in EμR pre–B cells when compared with that in WT pre–B cells, which is consistent with the findings that the Jκ region is accessible in EμR pre–B cells (Fig. 5 E).

The Jκ region becomes accessible in EμR pro–B cells and remains so in pre–B cells
To understand the mechanisms by which EμR activates Igk rearrangement at the pro–B cell stage, we analyzed k germline transcription in pro– and pre–B cells sorted from WT and EμR BM. A real-time quantitative PCR strategy was designed to determine the amount of GT initiating from promoters upstream of the Jκ gene segments (κ0GT) using primers immediately upstream of Jκ1 and within the Cκ exon. mRNA levels of β-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 EμR pro–B cells, our analysis indicated an approximate threefold increase in the amount of κ0 transcripts in EμR pro–B cells when compared with WT pro–B cells (Fig. 6 A). The amount of κ0 transcript in EμR pre–B cells was similar to that in WT pro–B cells, suggesting that the Igk locus remains in an open configuration at the Jκ region in EμR 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 Jκ and Cκ exons (JκCκ intron) in EμR and WT pro– and pre–B cells. The methylation status at an HhaI site residing within the JκCκ intron just downstream of the Jκ5 gene segment has been frequently analyzed as an indicator of the methylation status of the JκCκ 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, JκCκ demethylation was substantially increased in the pro–B cells of EμR mice (Fig. 6 C). More strikingly, the majority of Igk loci appeared to be demethylated in the EμR pre–B cells (Fig. 6 C). Therefore, this assay demonstrates that EμR has the ability to induce demethylation of the JκCκ 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 JκCκ 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 EμR 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 EμR pre–B cells, the JκCκ 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 MιEκ−/− B cells, suggesting the possibility that the hypomethylation observed in EμR loci is caused by the absence of MιEκ (23). However, similar to the WT allele, the MιEκ− 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 EμR locus is caused by the presence of EμR, not the absence of MιEκ. In conclusion, our findings indicate that EμR can promote robust chromatin remodeling of the JκCκ region in both pro– and pre–B cells.

The Vκ regions are accessible in EμR 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 Vκ germline transcription (VκGT) predicts their accessibility to V(D)J recombinase (1), we examined germline transcription of five Vκ gene segments that spread throughout the locus in EμR pro– and pre–B cells (Fig. 7 A). The amount of VκGT was quantitated by real-time PCR using primers annealing to the transcribed regions downstream of the Vκ RSS, which are deleted upon rearrangement and, thus, only amplify transcription from unrearranged Vκ promoters (Fig. 7 B). In pooled EμR pro–B cells, the Vκ germline transcription of three of five Vκ genes was increased in EμR pro–B cells when compared with WT pro–B cells (Fig. 7 C). Thus, consistent with the increased rearrangement of the EμR allele in pro–B cells, EμR appears to increase the accessibility of Vκ genes in pro–B cells.
To understand the basis for the inefficient Igk rearrangement observed in EμR pre–B cells, we also analyzed germline transcription of five Vκ segments in EμR pre–B cells. Because the percentage of Vκ in germline configuration is likely to be different between WT and EμR pre–B cells, the ratio of the amount of each germline Vκ in EμR pre–B cells versus WT pre–B cells was analyzed by quantitative real-time PCR using primers that are specific to each unrearranged Vκ (Fig. 7 E). By normalizing the levels of Vκ GT to the amount of germline Vκ, germline transcription of four of the five Vκ genes examined was markedly reduced in EμR pre–B cells when compared with that in WT pre–B cells, suggesting a reduced Vκ accessibility in EμR 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 Vκ segments in pooled WT and EμR pre–B cells by chromatin immunoprecipitation (ChIP) assays using an antibody against acetylated histone H3. To specifically detect only germline Vκ gene segments, we used the same primer sets that were used to analyze Vκ GT to determine the amount of germline Vκ in both immunoprecipitated chromatin and total input chromatin. In pooled pre–B cells sorted from five EμR and five age-matched WT littermates, histone acetylation of the analyzed Vκ gene segments was generally reduced (Fig. 7 F). As a control, we also examined acetylation at the Cκ exon and found equivalent levels of acetylation between EμR and WT pre–B cells, which is consistent with the observation that the Jκ/Cκ region remains open in pre–B cells (Fig. 7 F). Together with the reduction in Vκ GT, these data indicate that the inefficient Igk rearrangement observed in EμR pre–B cells is caused by the reduced accessibility of Vκ regions to the V(D)J recombinase.

The reduction in Vκ accessibility observed in EμR pre–B cells could be caused by the presence of Eμ and/or the absence of MiEκ. To distinguish between these possibilities, we analyzed Vκ germline transcription in pre–B cells sorted from MiEκ−/− mice. Germline transcription of most Vκ genes analyzed was considerably reduced in MiEκ−/− pre–B cells compared with that in WT pre–B cells, indicating that MiEκ is required for the accessibility of Vκ in pre–B cells (Fig. 7 G). In contrast to that in EμR pre–B cells, κ′GT was dramatically reduced in MiEκ−/− pre–B cells (Fig. 7 G). Vκ GT was normal in 3′κ′κ−/− pre–B cells but, consistent with previous studies (22, 23), κ′GT was reduced (Fig. 7 H). Therefore, MiEκ plays an important role in promoting the accessibility of both Vκ and Jκ regions in pre–B cells, and Eμ can compensate for MiEκ in opening up the Jκ region but not the Vκ region in EμR 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 pre–B cells, whereas the rearrangement of IgL chain genes occurs primarily in pre–B cells. Our findings indicate that EμR replacement greatly increases Igk

**Figure 7. Analysis of Vκ gene accessibility in Igk mutant mice.**

[A] Map of Igk locus. Locations of Vκ genes are represented by vertical lines. The six Vκ genes analyzed are labeled above and are represented with longer lines. The transcriptional orientation of each of the six Vκ genes is indicated by arrowheads below. The Jκ−Cκ region is represented by a broken line. (B) Real-time PCR strategy to quantitate the amount of Vκ germline transcription (Vκ GT). A generic map of unrearranged Vκ gene segments is shown with leader (L) and Vκ exons (boxes) and the RSS sequence (triangles). Forward (f) and reverse (r) primers are indicated by arrowheads. (C) Vκ GT level of five Vκ genes was analyzed by quantitative real-time PCR and normalized to CD19 mRNA levels. The thick horizontal line at the relative Vκ GT value of five indicates a change in scale. (D) Vκ GT in WT and EμR pre–B cells. All Vκ GT levels were normalized to the levels of CD19 mRNA and the relative amount of unrearranged Vκ alleles in EμR pre–B cells versus that in WT pre–B cells, as shown in E. (C and D) The y axis represents the ratio of Vκ GT in EμR pre–B cells versus that in WT pre–B cells. Error bars represent SD. (F) Histone acetylation of Vκ in pools of WT and EμR 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 Vκ genes are the same as those used for the Vκ GT analysis, and the Cκ, primers are the same as used in Fig. 5 B. (G and H) Germline transcription of Vκ and Jκ regions in MiEκ−/− (G) and 3′κ′κ−/− (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 EμR pro–B cells, indicating that Eμ 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 pro–B cells. Analysis of B cell development and Igκ rearrangement suggests that Igκ rearrangement is not efficient in EμR pro–B cells. In further support of this notion, the percentage of $V_{J_{\kappa}}$ junctions containing N nucleotides is similar in EμR pro–B and pro–B cells, indicating that the majority of the Igκ rearrangements detected in EμR pro–B cells have occurred in pro–B cells. In addition, the level of RAG-induced SE breaks is markedly reduced in EμR pro–B cells. Therefore, Eμ promotes V(D)J recombination in pro–B cells but cannot support efficient V(D)J rearrangement in pro–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 EμR pro–B cells, the inefficient rearrangement of the EμR allele in pro–B cells is not caused by the accessibility of the $J_{\kappa}$ region to the V(D)J recombinase. In contrast, germline transcription and histone acetylation of unarranged $V_{\kappa}$ gene segments are noticeably reduced in EμR pro–B cells, indicating that accessibility of the $V_{\kappa}$ region is reduced in EμR pro–B cells. This scenario is remarkably similar to the IgH locus in normal pro–B cells, where the $V_{H}$ region becomes inaccessible but the $D_{H}$ region remains accessible (35). As germline transcription of $V_{\kappa}$ and $J_{\kappa}$ is considerably reduced in Eμκ−/− pre–B cells, the reduced accessibility of $V_{\kappa}$ in EμR pre–B cells is caused by the lack of MiEκ. Therefore, Eμ and MiEκ maintain the sequential rearrangement of the Igκ 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 Eμ and MiEκ shows that μA and μB sites are only present in Eμ. The binding of PU.1 and ETS family proteins to μA and μB sites promotes chromatin accessibility to endonucleases (60). In addition, although two E2A-binding E boxes, which are important for activating Igκ rearrangement, are present in MiEκ (40), three E2A-binding E boxes are present in Eμ (61). The unique combination of functional motifs within Eμ might account for its activities in activating V(D)J recombination in pro–B cells.

It remains unclear why Eμ cannot compensate for MiEκ function in promoting the accessibility of $V_{\kappa}$ regions in pro–B cells. Most of the known protein-binding sites within MiEκ are also found in Eμ. One exception is the NF–κB–binding site. However, deletion of this site has no considerable impact on Igκ rearrangement (40). Both MiEκ and Eμ contain 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 Eμ and MiEκ function differently in promoting $V_{\kappa}$ accessibility in pro–B cells. Although neither the two MARs of Eμ nor the MAR of MiEκ is required for rearrangement (66, 67), increased premature Igκ rearrangement in pro–B cells has been detected in mice with a deletion of MAR of MiEκ (67, 68). It is possible that certain unidentified elements within MiEκ are required for promoting the accessibility of $V_{\kappa}$ in pro–B cells. Alternatively, some elements within Eμκ, such as μNR, might dominantly suppress its activity in promoting accessibility in V regions in pro–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 Eμκ and MiEκ 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 EμR and MiEκ−/− precursor B cells will help to define the functions of intronic enhancers in regulating such processes. In summary, the IgH and Igκ 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 EμR ES cells and knockin mice.** Eμκ was cloned into pBluescript and inserted into a targeting construct that was used previously to knockout the Igκ intronic enhancer (21). Eμκ was inserted into the Sall site upstream of the floxed neomycin resistance gene under control of the PGK promoter (f-PGK-neor), the same insertion site previously used to reintroduce mutant MiEκ 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 μg/ml G418 and 1 μM gancyclovir to select for integration by homologous recombination. Positive clones were first screened by PCR and then by Southern blotting, digesting with EcoR1 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 PGκ-neor 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 EμR, produces an 11.0-kb fragment. Positive clones were then subcloned and rescreened to ensure purity. EμR ES cells were injected into mouse blastocysts to produce chimeric mice, which were bred and screened for germline transmission of the EμR 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 NH4Cl, 1 mM KHCO3, and 0.1 mM Na2EDTA, pH 7.0) for 5 min at room temperature. 1 million cells were pretreated with 1 μg FcBlock (BD Biosciences) and stained in 100-μl 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 μg/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 IgG-FITC (BD Biosciences), streptavidin-APC (eBioscience), and streptavidin-PECy5 (eBioscience). To obtain purified pre– 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+, IgM+, and CD43+) and pre–B cells (B220+, IgM+, and CD43+) were sorted using a FACSVantage Cell Sorter (Becton Dickinson). κ+ spleen cells were sorted by negative selection using the MACS system (Miltenyi Biotec), anti–IgG–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+.

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+ spleen 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 ΔCt 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 by using the degenerate Vκ primer (VκD) 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 described previously (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 phosphomager (Storm; GE Healthcare) as previously described (23).

Cloning and sequencing of Vκ+λ rearrangements. Rearranged Igk alleles were amplified using KOD Hotstart Polymerase (Calbiochem) according to the manufacturer’s instructions. A degenerate Vκ primer with a BamHI restriction sequence inserted (VκD Bam) and a primer that anneals between Jκ1 and Jκ2 with an EcoRI restriction sequence added (Jκ1 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 Vκ 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 Vκ or Jκ 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 ACGGGCTGG, where the underlined sequences correspond to Vκ (left) and Jκ (right), GGC would represent inserted nucleotides. Because the inserted GG nucleotides are complementary to the Vκ end, they would be classified as P nucleotides. The inserted C nucleotide, which is not complementary to either Vκ or Jκ 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. Schlissel’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 Jκ1 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, 55°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 Li2 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 precleaving, immunoprecipitation was performed overnight at 4°C using 3 μl of acetylated 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 MiEκ−/− BM and spleen by flow cytometry. Fig. S2 shows the analysis of B cell populations in EqR × Iκκ−/− BM and spleen. Fig. S3 shows the analysis of methylation in MiEκ−/− 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.
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