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The mouse Igκ locus has three known transcriptional enhancers: the matrix association region/intronic enhancer, the 3′ enhancer (E3′), and the further downstream enhancer (Ed). Previous studies have shown that both matrix association region/intronic and E3′ enhancers are required for maximal gene rearrangement of the locus, and that E3′ is also required for maximal expression and somatic hypermutation (SHM). To functionally elucidate Ed in vivo, we generated knockout mice with a targeted germline deletion of Ed. Ed deleted homozygous mice (Ed−/−) have moderately reduced numbers of Igκ expressing B cells and correspondingly increased numbers of Igκ expressing B cells in spleen. Ed−/− mice also have decreased Igκ mRNA expression in resting and T-cell-dependent activated splenic B cells and reduced Igκ chains in sera. However, our analysis indicates that Igκ gene rearrangement is normal in Ed−/− mice. In addition, our results show that Ed−/− mice exhibit reduced SHM in the Igκ gene J-C intronic region in germinal center B cells from Peyer’s patches. We conclude that Ed positively regulates Igκ gene expression and SHM, but not gene rearrangement. The Journal of Immunology, 2008, 180: 6725–6732.

During B cell development, the IgH chain gene rearranges first, by sequential D-J and then by V(D)J joining, leading to the pro- and pre-B cell stages of development, respectively (1). The Igκ locus is poised for rearrangement in pre-B cells, and, upon appropriate signaling, one of the 95 Vκ genes is semi-randomly selected for recombination to a J region (for review of recombination, see Ref. 2), evidently by the RAG proteins first making single-stranded nicks at Vκ recombination signal sequences (RSS)3 followed by a capture model for synapsis (3). The Igκ locus thus offers the opportunity to visualize changes in chromatin structure that may precede gene rearrangement and transcriptional activation during B lymphocyte differentiation, as well as those remodeling events that may accompany or be a consequence of gene activation (4 and Refs. within). In addition, the mouse Igκ gene locus has provided a paradigm for investigating site-specific recombination and tissue-specific transcriptional regulation (reviewed in Ref. 5); somatic hypermutation (SHM) (reviewed in Ref. 6); DNA methylation (7); higher-order chromatin organization (8); and nuclear organization and allelic exclusion (reviewed in ref. 9).

Several previous studies have identified a number of cis-acting regulatory elements in the mouse Igκ gene locus. All of these elements reside in a 32 kb segment near or within the Jκ-Cκ region toward the 3′ end of the 3.2 mega base locus, except for Vκ gene promoter elements and their RSSs (10) and the recombining sequence (11). These include: a recombination silencer (12), two germline promoter elements (13, 14), KI-KII sequences (15), RSSs associated with J regions (16), a nuclear matrix association region (MAR) (17), an intronic enhancer (Ei) (18), a transcription terminating region (19), a 3′ enhancer (E3′) (20), and further downstream enhancer (Ed) (4).

The functional significance of several of the above cis-acting sequences has been addressed by creating their targeted deletion from the native locus in cell lines or mice. Deletion of a germline promoter, or KI-KII sequences, or both, results in lower levels of gene rearrangement (15, 21, 22). Deletion of the MAR from the mouse germline down-regulates SHM and mildly stimulates precocious Vκ-Jκ joining (23), whereas its deletion in a pre-B cell line results in hyper-recombination (24). Deletion of either MAR/intronic enhancer (MEi) or E3′ severely reduces, but does not abolish, Igκ gene rearrangement (25, 26), whereas deletion of both enhancers nearly completely blocks recombination, indicating that each element has a redundant, but critical, role in regulating gene rearrangement in the locus (27). Deletion of E3′ also results in reduced SHM (28), in contrast to an earlier report (29), and also reduced Igκ gene expression (26, 28).

Several years ago, our laboratory discovered a third enhancer in the mouse Igκ locus, initially from an analysis of B cell-specific Dnase I hypersensitive sites in chromatin (4). This enhancer is in the far downstream region, some 8 kb 3′ of the E3′ element, and was coined Ed. Because the mouse Ed sequence is heavily conserved in the human genome and shares NF-κB and E2A binding sites, we decided to elucidate its function in the native locus by creating a targeted deletion in the mouse germline, and report here the phenotypes resulting from this deletion. We demonstrate that Ed plays no role in regulating recombination, a result consistent with our previous findings that the element exhibits hypersensitive sites in plasmacytoma cells but not in pre-B cells (4). Rather, we...
have found that Ed is required for maximal Igκ gene expression in resting and T cell-dependent stimulated splenic B cells. We further demonstrate the SHM is down-regulated in germinal center (GC) B cells from Peyers’ patches of these Ed−/− knockout mice.

Materials and Methods

Generation of Ed knockout mice

Homology arms of the Ed targeting vector were genomic DNA fragments from a λ phage clone containing the mouse Ed genomic locus, which was isolated by screening a 129SVJ mouse genomic library (Stratagene). The Ed targeting vector was constructed as follows: first, a PGK-neoIII gene fragment flanked by two loxP sites was excised from the NL-24 vector (30) and inserted into phleomycin II KS (Stratagene), and a Ndel site was introduced into the PBS vector by inserting a polylinker. Next, a 3.6 kb Ndel 129SVJ genomic DNA fragment as a 3′ homology arm was cloned into the Ndel site. And then, a 1.8 kb HindIII 129SVJ genomic DNA fragment corresponding to the 5′ homology arm was cloned into a HindIII site. Finally, the 1.2 kb HindIII Ndel Ed enhancer-containing fragment was PCR amplified using 129SVJ genomic DNA as template. A loxp site was introduced into this PCR fragment using the primers: Eddu:CATTAGCGGCCGCATAACTTCGTATAGCAT and Eddd: CATTAGCCGGCGCATATACTTGATGTCACGTTAG. The PCR used 35 cycles, each cycle consisted of 1 min at 94°C, 1 min at 59°C, and 2 min at 68°C. Then, the PCR-amplified Ed fragment was cloned into the Ed targeting vector. The SM-I (129/SvEvTac) embryonic stem cell line was cultured in RPMI 1640 medium supplemented with 10% FBS, penicillin/streptomycin, t-glutamine, and 50 μM 2-ME at 10% cells/ml, and then were stimulated with 20 μg/ml LPS (Sigma-Aldrich), 2.5 μg/ml anti-CD40 Abs (HM40-3; BD Pharmingen) or 25 μg/ml anti-CD40 Abs and 10 ng/ml recombinant mouse IL-4 (BD Pharmingen) for 4 days.

Flow cytometry

Single-cell suspensions were prepared from bone marrow and spleen. Cells (1 × 10⁶) were stained with an optimal concentration of FITC-, PE-, or biotin-conjugated Abs in 100 μl of PBS/2%FCS for 20 min on 4°C. Stained cells were analyzed by a FACSCalibur (BD Biosciences). The following Abs were used: FITC-anti-B220 (RA3–6B2), anti-λ (R26–46), anti-CD21 (7G6) (BD Pharmingen); PE-anti-Igk (187.1), anti-B220 (RA3–6B2) (BD Pharmingen); biotinylated-anti-IgM (II/41), anti-CD138 (syndecan-1) (281-2I) (BD Pharmingen); and FITC-anti-IgD (11–26), allophycocyanin-anti-CD23 (2G8) (Southern Biotechnology Associates). The biotin conjugates were revealed with streptavidin-phycoerythrin (Southern Biotechnology Associates). Only cells residing in the lymphocyte gate were analyzed. Dead cells were excluded by size and forward scatter gating. Data were analyzed with CellQuest (BD Biosciences) or FlowJo (Tree Star) software.

Northern blotting analysis of Igκ expression

Single-cell suspensions were prepared and 2 × 10⁶ spleen cells from wild-type (WT) or Ed−/− mice were incubated with an optimal concentration of biotinylated anti-κ Abs and purified by using MACS MS separation columns according to the manufacturer’s instructions. κ cell populations were found to be >95% pure by FACS analysis (data not shown). Splenic activated and resting B cells were purified by Percoll gradient centrifugation as described (32). Total RNA was isolated from purified cells by extraction with Trizol reagent (Invitrogen) and a 6.2 kb band derived from the floxed allele were detected by probe B (Fig. 1), residing outside of the targeting vector, were used to hybridize NcoI-digested embryonic stem (ES) cell genomic DNA. Three independent Ed targeted clones were injected into C57/BL6 blastocysts. Chimeric mice were bred with C57/BL6 mice to obtain germline transmissible mice. Only germline transmissible mice were bred with Cre recombinase expressing MORE mice (31) to obtain Ed and neo deletion mice. All mice were used in accordance with protocols approved by the UT Southwestern Medical Center Institutional Animal Care and Use Committee.

Northern blotting was performed to determine fragment sizes. Probe A or B (Fig. 1), residing outside of the targeting vector, were used to hybridize NcoI-digested embryonic stem (ES) cell genomic DNA. Three independent Ed targeted clones were injected into C57/BL6 blastocysts. Chimeric mice were bred with C57/BL6 mice to obtain germline transmissible mice. Only germline transmissible mice were bred with Cre recombinase expressing MORE mice (31) to obtain Ed and neo deletion mice. All mice were used in accordance with protocols approved by the UT Southwestern Medical Center Institutional Animal Care and Use Committee.

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B cell culture

Single-cell suspensions of spleens from WT and Ed−/− mice were incubated with an optimal concentration of biotinylated anti-CD43 (Ly-48; BD Pharmingen) Ab and biotinylated-anti-κ (JC5-1; Southern Biotechnology Associates) Ab. Then MACS MS separation columns (Miltenyi Biotec) were used to deplete CD43+ and λ5+B cells. κ enriched B cells were cultured in RPMI 1640 medium supplemented with 10% FBS, penicillin/streptomycin, t-glutamine, and 50 μM 2-ME at 10% cells/ml, and then were stimulated with 20 μg/ml LPS (Sigma-Aldrich), 2.5 μg/ml anti-CD40 Abs (HM40-3; BD Pharmingen) or 25 μg/ml anti-CD40 Abs and 10 ng/ml recombinant mouse IL-4 (BD Pharmingen) for 4 days.

ELISA analysis

Sera collected from 10–16-wk-old WT and Ed−/− mice were analyzed for Igκ and Igλ chain concentrations by sandwich ELISA. PVC microtitrater plates (DYXEN Technologies) were coated with polyclonal goat anti-mouse Ig (H+L) (Southern Biotechnology Associates) at 10 μg/ml as the
capturing Ab. After blocking for 2 h with 3% BSA in PBS at room temperature, diluted sera were added and incubated at 4°C overnight. Plates were subsequently washed three times and incubated with either goat anti-mouse κ-HRP or goat anti-mouse λ-HRP (Southern Biotechnology Associates) for 2 h. After washing three times, plates were developed with the ABTS substrate (Southern Biotechnology Associates) following the manufacturer’s instructions. Purified mouse IgG3, κ isotype standard and purified mouse IgG2a, λ isotype standard (BD Pharmingen) were used to quantify ELISA data.

Southern blotting and quantitative PCR analysis of Igk V-J rearrangement

Single-cell suspensions containing 2 × 10³ splenic or bone marrow cells from WT or Ed−/− mice were incubated with an optimal concentration of biotinylated-anti-κ Ab or biotinylated-anti-B220 Ab, and κ⁺ cells or B220⁺ cells were purified by using MACS MS separation columns. The purified cells were lysed in lysis buffer with protease K. Genomic DNA was then purified by phenol/chloroform extraction, followed by ethanol precipitation. A total of 10 μg of genomic DNA was digested with BanHI/EcoRI and transferred to Zeta-probe GT genomic membrane (Bio-Rad) after electrophoretic separation on agarose gels. Prehybridization and hybridization were performed at 65°C with ExpressHyb hybridization buffer (Clontech). The membranes were hybridized with [α-³²P]dCTP-labeled C kappa (Molecular Dynamics). A quantitative Igk V-J rearrangement PCR assay was performed as described (23, 27). Briefly, the Vκk-Jκk rearrangement products were PCR amplified by using the Platinum Taq high fidelity DNA polymerase (Invitrogen) with a degenerate Vκ primer (Vκd) (33) and a MAR35 primer (27). PCR cycles were as follows: 94°C denaturation for 3 min, followed by 27 cycles at 94°C for 1 min, 60°C for 1 min, 68°C for 3 min, and a final 5 min extension at 68°C. The amount of genomic DNA used in each PCR was controlled by the c-myc PCR, which specifically amplifies a 0.7-kb c-myc genomic fragment (26). PCR products were resolved by electrophoresis in 1% agarose gels and transferred to nylon membrane (Bio-Rad). The membranes were hybridized with a probe within the Jκ to MEI region (Fig. 7D). Membranes were exposed to PhosphorImaging screens, and images were analyzed using ImageQuant software (Molecular Dynamics).

Cell sorting and SHM analyses

Single-cell suspensions prepared from Peyer’s patches were stained with PE-anti-B220 and FITC-PNA (Vector Laboratories). B200⁺ PNAhighgc cells and B220⁺ PNAlowgc cells were sorted on a MoFlo machine (Dako). For the Jκ-Cκ intronic region SHM analysis, genomic DNA was purified from sorted WT and Ed−/− GC B cells. The Jκ-Cκ intronic regions from rearranged genes were PCR amplified by using the Platinum Taq high fidelity DNA polymerase (Invitrogen) with a degenerate Vκ primer (33) and a reverse primer located −600 bp downstream of the Jκ5: AGCGAAACACTTTACATGAGGACAAAAGAGGAGAAC. The PCR used 35 cycles, each cycle consisted of 30 s at 94°C, 30 s at 56°C, and 1 min at 68°C. Gel purified Vκ-Jκ5 PCR products were cloned into the PGEM-T vector (Promega). Vκ-Jκ5 clones were identified and sequenced by use of a T7 primer. Sequences were aligned with the mouse Jκ5 downstream sequence using the Vector NTI (Invitrogen) AlignX program, and mismatches were scored as mutations in the 500 bp region downstream of Jκ5.

Results

Generation of Ed−/− mice

To delete Ed from the native locus, we first constructed a targeting vector in which the entire 1.2 kb enhancer was replaced with Ed and a PGK-neo' gene flanked by loxP sites (Fig. I, A and B). ES cells were transfected with this linearized construct, and several clones that exhibited site-directed integration were obtained after screening by PCR and Southern blotting (Fig. 1C). Three independent targeted ES cell clones were used to generate chimeric mice lines. Chimeric mice were bred with C57/BL6 mice to obtain germline transmission. To delete Ed and the PGK-neo' gene (Fig. 1D), we bred germline transmissible mice with Cre recombinase expressing MORE mice (31). Ed-deleted heterozygous mice were interbred to obtain Ed-deleted homozygous mice, which are referred to hereafter as Ed−/− mice. Various stages of the targeting and Ed deletion were confirmed by Southern blotting. Genomic DNA was digested with NcoI and hybridized with either probe A or B, which generated a 9.9 kb WT band, a 8.6 kb Ed-deleted band, and a 5.0 or 6.2 kb PGK-neo' gene replacement band detected by probe A and B, respectively (Fig. 1, E and F).

Ed−/− mice exhibit a modest decrease in Igk expression in splenic B cells

We found that Ed−/− mice exhibited no significant differences in bone marrow and spleen B cell numbers compared with those of their WT littermates or age-matched WT mice (data not shown). However, Ed−/− mice exhibited a moderate decrease of Igk⁺ B cells in spleen compared with WT mice (Fig. 2A; 43 ± 1% vs 48 ± 2%, as percentages of Igk⁺ B cells among total lymphocytes, n = 6, p < 0.01, Student’s t test). In contrast, the percentage of Igλ⁺ B cells was increased by ~2-fold (Fig. 2A; 5.3 ± 1.5% vs 2.8 ± 0.5%, as percentages of Igk⁺ B cells among total lymphocytes, n = 5, p < 0.01, Student’s t test). These changes resulted in a moderate decrease of Igk/λ ratio, ~8.1/1 in Ed−/− mice as opposed to 17.1/1 in WT mice. Because the percentages of Igk⁺ and Igλ⁺ cells in bone marrow were not different between Ed−/− and WT mice (Fig. 2B), we conclude that the alteration in the distributions of Igk⁺ and Igλ⁺ cells occurs in the periphery. Furthermore, FACS analysis showed that the percentages of pro- and pre-B cells (B220⁺/lowIgM⁻), newly generated immature B cells, and recirculating B cells (B220⁺/IgM⁺) in bone marrow exhibited no significant differences between Ed−/− and WT mice (data not shown). We also observed a similar decrease of Igk⁺ cells in gated splenic IgM⁺ cells in Ed−/− mice (Fig. 2C). And the mean fluorescence intensity of Igk expressing B cells in Ed−/− mice was decreased compared with those of WT (Fig. 2C; 1138 ± 5.0 vs 1268 ± 21.5, n = 3, p < 0.01, Student’s t test). These results indicate that the decreased Igk⁺ cells and altered Igk/λ ratio in the spleen of Ed−/− mice must be primarily established during the maturation of B cells in the spleen, and not during the generation of B cells in bone marrow at the level of recombination.

Ed−/− mice exhibit little differences from WT mice in marginal zone, follicular, and transitional splenic B cells and in plasma cell formation

To further investigate the effect of Ed deletion on the development of B cells, we analyzed the percentages of transitional B cells, follicular mature B cells, and marginal zone B cells in the spleen by FACS. Ed−/− mice exhibited a slight increase in T1 (IgMhighIgD⁻) and T2 (IgMhighIgDhigh) transitional B cells, and a slight decrease in follicular mature (IgM⁺IgD⁻) B cells relative to the corresponding cell distributions exhibited by WT mice (Fig. 3A). We also found that the percentage of marginal zone B cells was normal in Ed−/− mice (Fig. 3B). Previous studies in our laboratory demonstrated that Ed exhibits a plasmacytoma cell-specific DNase I hypersensitive site (4). To address the question of whether plasma cell development is impaired in Ed−/− mice, we analyzed the percentages of B220⁺/CD138⁺ plasma cells in spleen by FACS, and we found there was no significant difference between WT and Ed−/− mice (0.27 ± 0.13% vs 0.29 ± 0.05%, respectively; data not shown). When cultured with LPS in vitro, splenic B cells proliferate and differentiate into Ig-secreting cells (34). We found that the percentage of B220⁺/CD138⁺ Ig-secreting cells was almost the same between LPS-treated WT and Ed−/− splenic B cells (Fig. 3C; 13 ± 1.5% vs 12 ± 0.6%). Northern blot analysis showed that the level of Igk mRNA expression in these purified Ed−/− B220⁺/CD138⁺ Ab-secreting cells was also normal (data not shown). In summary, these data indicate that the splenic B cell distributions and plasma cell formation are approximately normal in Ed−/− mice.
Ed is required for normal Igκ expression

Because we found that cell surface Igκ expression levels were decreased in Ed−/− splenic Igκ⁺ B cells (Fig. 2C), we assayed for the steady-state levels of Igκ mRNA by Northern blot analysis. RNA samples from splenic Igκ⁺ cells were hybridized with Cκ, Cα, and β-actin probes. Then, Igκ RNA levels were normalized to the total amount of β-actin RNA in the corresponding lanes. Igκ RNA levels of Ed−/− B cells were decreased compared with those of WT (Fig. 4, A and C; 67 ± 8.2% vs 100 ± 5.6%, n = 5, p < 0.01, Student’s t test). Furthermore, when we normalized Igκ RNA levels to those of Igκ RNA, we found a similar decrease in Ed−/− B cells compared with those of WT (Fig. 4A; 51 ± 22.8% vs 100 ± 7.7%, n = 3, p < 0.01, Student’s t test). We also compared Igκ RNA levels of Ed−/− and WT B cells after 4 days of LPS stimulation in culture. We found Ed−/− and WT B cells expressed similar levels of Igκ RNA after LPS stimulation (Fig. 4, B and D; 105 ± 13% vs 100 ± 8.7%, n = 5, p = 0.51, Student’s t test). To further determine whether Igκ RNA levels were decreased in either activated and/or resting B cells, we purified activated and resting B cells from spleen by use of Percoll gradient centrifugation and analyzed the Igκ RNA levels by Northern blotting as above. We found Igκ RNA levels were decreased ∼50% in Ed−/− resting B cells compared with those of WT. In contrast, when compared with resting B cells, the reduction of Igκ RNA levels in Ed−/− activated B cells was less dramatic (Fig. 4E). We also analyzed sera for Igκ chain protein levels by ELISA. The sera...
Igκ chain levels were decreased in Ed−/− mice compared with those of WT (Fig. 5; 15.3 ± 6.4 vs 22.5 ± 6.7 mg/ml, n = 10, p = 0.02, Student’s t test). A corresponding increase in sera Igλ chain levels was observed in Ed−/− (Fig. 5; 1.46 ± 0.56 vs 0.91 ± 0.40 mg/ml, n = 10, p = 0.02, Student’s t test). In summary, these data indicate that Igκ expression is impaired in splenic B cells lacking the Ed enhancer.

Igκ expression is impaired in T cell-dependent stimulated Ed−/− splenic B cells

Previous studies have shown that Igκ expression is normal in E3’ enhancer knockout splenic B cells after stimulation with LPS (26). However, Igκ expression was decreased in these cells if activated by BCR or T cell-dependent stimulation, such as anti-IgM and/or anti-CD40 Ab (28). To test whether Ed is required for normal Igκ expression in a T cell-dependent stimulated immune response, we stimulated splenic B cells with anti-CD40 Ab or anti-CD40 Ab plus IL-4. Northern analyses revealed that Igκ mRNA levels were decreased in anti-CD40 activated Ed−/− splenic B cells compared with those of WT (Fig. 6, A and C; 66 ± 4% vs 100 ± 15%, n = 3, p = 0.02, Student’s t test). Similar results were found in anti-CD40 plus IL-4 activated Ed−/− splenic B cells (Fig. 6, B and D; 75 ± 7% vs 100 ± 9%, n = 3, p = 0.01, Student’s t test). Furthermore, when we normalized the Igκ RNA levels to those of Igμ, we found a similar decrease in Ed−/− B cells compared with those of WT (Fig. 6, A and B, and data not shown). We also found that the percentages of B220+/CD138+ Ig-secreting cells were...
The amount of genomic DNA template in PCR. PhosphorImager analysis of the resulting Southern blot. The V region of genomic DNA was normalized by the c-myb probe. In agreement with a previous report (35), the level of germline fragments was ~29% in Igκ− splenic B cells (Fig. 7B; WT), very similar to the levels seen in the corresponding cells from Ed−/− mice (Fig. 7B; 27 ± 3% vs 29 ± 9%, n = 3, p = 0.65, Student’s t test). Related assays on bone marrow B cells also revealed very similar levels of germline fragments from WT and Ed−/− mice that were not statistically different (Fig. 7C; 60 ± 11% vs 51 ± 11%, n = 3, p = 0.33, Student’s t test). To examine the possibility that Jκ region usage and secondary VκJκ rearrangement levels might be altered in Ed−/− mice, we used a quantitative PCR assay to analyze VκJκ rearrangement products in purified splenic Igκ+ and bone marrow B cells. The results revealed that the relative frequencies of Jκ region usage were similar in WT and Ed−/− samples (Fig. 7E). We conclude that Igκ gene rearrangement is unaffected in Ed−/− mice.

**Igκ locus rearrangement is normal in Ed−/− splenic and bone marrow B cells**

To further investigate the mechanism responsible for decreased splenic Igκ+ cells in Ed−/− mice, Southern blotting was used to assay for VκJκ rearrangement levels in purified splenic Igκ+ and bone marrow B cells. Electrophoretically resolved genomic DNA from purified cells that had been digested with EcoRI plus BamHI was transferred and hybridized with Cκ and c-myb probes. The amount of EcoRI plus BamHI-digested DNA was normalized by the c-myb signal. Igκ germline (c/c-c-myb) ratio in ES cells was set as 100%. The % c/c for each sample was calculated as: (% c/c-c-myb in samples)/(c/c-c-myb in ES cells) × 100%. Bone marrow B220+ cells genomic DNA was analyzed as described in B and C. Data are representative of at least two independent experiments. D, Schematic diagram of the quantitative PCR assay used for VκJκ rearrangement. The positions of VκD and MAR35 primers are indicated by the arrows, and the probe used for the Southern blot is indicated by a thick line. E, Analysis of Jκ regions usage in splenic κ+ and bone marrow B cells. VκJκ rearrangement PCR products were electrophoretically separated on agarose gels, and the intensities of VκJκ to VκJκ bands were quantitated by PhosphorImager analysis of the resulting Southern blot. The VκJκ/VκJκ ratio of WT samples was set as 100%, and the ratio of Ed+/− and Ed−/− samples are shown in the figure. The Southern blot results of PCR amplification of c-myb are shown at the bottom, which were used to control for the amount of genomic DNA template in PCR.

**SHM is decreased in the J-C intronic region of Ed−/− GC B cells**

The E box motif has been reported to enhance SHM (36, 37). In addition, the Igκ transcription efficiency has also been correlated with levels of SHM (6). Because Ed contains two E box sites and we also observed reduced Igκ expression in Ed−/− B cells and T cell-dependent activated B cells, we investigated the possibility that SHM might be decreased in Ed−/− GC B cells. We purified B220+ PNAhigh GC B cells from Peyser’s patches of Ed−/− and WT mice by flow cytometry. Genomic DNA was isolated from these GC B cells and VκJκ rearrangements were amplified using high fidelity PCR. VκJκ rearrangement products were gel purified, cloned, and sequenced. We found that the mutation frequency of a 500 bp intronic region downstream of Jκ5 was 11.1 × 10−3 mutations per base in WT GC B cells. In contrast, only 4.3 × 10−3 mutations per base were detected in Ed−/− GC B cells (Fig. 8; p < 0.01). The overall mutation frequency in Ed−/− GC B cells

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**FIGURE 7.** Analysis of Vκ-Jκ rearrangement levels by Southern blotting and quantitative PCR. A, Schematic diagram of the Southern blot assay for Vκ-Jκ rearrangement. E = EcoRI; B = BamHI. B, Electrophoretically resolved genomic DNA from splenic Igκ+ cells that had been digested with EcoRI plus BamHI and transferred was hybridized with Cκ and c-myb probes. The amount of EcoRI plus BamHI-digested DNA was normalized by the c-myb signal. Igκ germline (c/c-c-myb) ratio in ES cells was set as 100%. The % c/c for each sample was calculated as: (% c/c-c-myb in samples)/(c/c-c-myb in ES cells) × 100%. C, Bone marrow B220+ cells genomic DNA was analyzed as described in B. B and C, Data are representative of at least two independent experiments. D, Schematic diagram of the quantitative PCR assay used for VκJκ rearrangement. The positions of VκD and MAR35 primers are indicated by the arrows, and the probe used for the Southern blot is indicated by a thick line. E, Analysis of Jκ regions usage in splenic κ+ and bone marrow B cells. VκJκ rearrangement PCR products were electrophoretically separated on agarose gels, and the intensities of VκJκ to VκJκ bands were quantitated by PhosphorImager analysis of the resulting Southern blot. The VκJκ/VκJκ ratio of WT samples was set as 100%, and the ratio of Ed+/− and Ed−/− samples are shown in the figure. The Southern blot results of PCR amplification of c-myb are shown at the bottom, which were used to control for the amount of genomic DNA template in PCR.
was 2.5-fold reduced compared with that of WT GC B cells. Among clones bearing mutations, the mutation frequency was 17.1 × 10⁻⁴ mutations per base in WT GC B cells, and only 6.6 × 10⁻³ mutations per base in Ed⁻/-/GC B cells (Fig. 8). The mutation frequency was also ~2.5-fold reduced in Ed⁻/-/GC B cells. Control experiments revealed that error frequency of the high fidelity PCR was only 0.13 mutations per kb, and the mutation frequency of WT B220⁺PNA⁺ B cells was <1 mutation per kb (data not shown). Furthermore, we found that the percentage of clones with no mutations was 43% in Ed⁻/-/GC B cells, but only 25% in WT GC B cells. In summary, these data indicate that Igκ SHM is decreased in Ed⁻/-/GC B cells from Peyer’s patches.

Discussion

The results of our study complete the picture on the individual roles of the three Igκ transcriptional enhancers in the locus with respect to gene rearrangement, transcription of rearranged genes, and SHM. Unlike MEi and E3', which each serve a redundant role in triggering V-J joining in the locus at the appropriate stage of B cell development (25–27), our results on Ed⁻/-/ mice reveal no defect in gene rearrangement, both by the numbers of Igκ⁺ cells in bone marrow and germline DNA content in bone marrow and spleen (Figs. 2 and 7). In addition, the fact that Igκ V-J rearrangement was essentially abolished in MEi and E3' double knockout mice also supports the conclusion that Ed by itself cannot trigger Igκ gene rearrangement (27). We have also found no defects in recombining sequence recombination in Ed⁻/-/ mice (data not shown). We conclude that the deficit of Igκ chains in sera (Fig. 5) and in the fraction of Igκ⁻ cells in the periphery (Fig. 2) in Ed⁻/-/ mice must be due to defects in B cell behavior after the cells have left the bone marrow.

We have previously noted that Ed forms a DNase I hypersensitive site in B cell chromatin in plasmacytoma but not pre-B cell lines (4), an observation also consistent with a functional role for Ed later in B cell maturation. These findings prompted us to measure the percentages of plasma cells in spleen and the production of Igκ-secreting cells upon stimulating splenic B cells with LPS. However, we found that there was no difference in plasma cell number between WT and Ed⁻/-/ cells (Fig. 3C). The deficit of Igκ chains in Ed⁻/-/ mice sera can’t be explained only by decreased Igκ⁺ cells in the periphery, because MEi⁻/-/ and E3'⁻/-/ knockout mice have more dramatic decreases in Igκ⁺ cells in the periphery than do Ed⁻/-/ mice, but Igκ chains in sera of MEi⁻/-/ and E3'⁻/-/ mice are at concentrations similar to those of WT (25–27). In conclusion, we still cannot rule out the possibility that Ed plays roles in Ab secretion in vivo. T cell-dependent and -independent immunization experiments are required to further elucidate the function of Ed in immune responses and/or Ab secretion.

With respect to transcription levels of rearranged genes in unstimulated B cells, previous studies have shown the MEi⁻/-/ mice have no defect in rearranged Igκ gene expression (25, 28). By contrast, lower levels of rearranged gene transcripts exist in splenic B cells of E3'⁻/-/ mice (26, 27), or upon their BCR or T cell-dependent stimulation (28), but not after LPS stimulation (26, 28). Ed⁻/-/ mice Igκ gene transcription levels closely fit the same patterns as those exhibited by E3'⁻/-/ mice, with moderately reduced cell surface Igκ chains and Igκ gene mRNA levels in splenic κ⁺ cells, serious deficits in resting splenic B cells transcript levels (Figs. 2C and 4, C and E), and in responsiveness to transcriptional activation by a T cell-dependent pathway (Fig. 6, C and D), but not by the LPS activation pathway (Fig. 4D). We found that in vivo activated Ed⁻/-/ splenic B cells only exhibited slightly reduced Igκ gene transcript levels, which can be explained by the fact that part of splenic B cells, marginal zone cells, can be activated by T cell-independent Ags (38). Our previous studies have shown that the Ed enhancer synergistically activates transcription in combination with other enhancers, like Ei (4), and that Ed forms complexes with Ei and E3' with the looping out of the intervening DNA in stimulated splenic B cells as well as plasmacytoma cell lines (8). Taken together with the results of the present investigation, it seems clear that E3' and Ed in particular must work together to optimally set up active chromatin loop domains and to drive high level Igκ gene expression during resting and activated B cell states.

We also studied Igκ transcript levels relative to those of Igκ in WT and Ed⁻/-/ mice in various B cell populations. Surprisingly, in cell populations where Igκ transcript levels were compromised in Ed⁻/-/ mice, no corresponding down-regulation was observed for Igκ transcript levels (Figs. 4 and 6). This raises the possibilities that overabundant Igκ chains may be cytoplasmic, or associated with up-regulated surrogate light chains on the cell surface, or that Igκ protein levels are stoichiometrically adjusted to be equimolar with Igκ chains by regulation of their translational efficiency.

Studies on cis-acting elements that are involved in specifying SHM in mice were first performed on rearranged Igκ transgenes that lacked Ed (39, 40). These studies found that both MEi and E3' were required for SHM and that the level of SHM correlated with the level of expression. Another more recent transgenic study, again with a construct lacking Ed, was able to disconnect the correlation between expression level and SHM, because upon mutation of E2A binding sites in the transgene, no affect on transcription was seen, but SHM went down by 4-fold (36). This result points to a role for certain transcription factors, and not simply to the levels of RNA polymerase II traversal, in the targeting of the mutator apparatus. The first experiments on SHM in Igκ enhancer knockouts in the native locus were performed in the Alt laboratory. By contrast to the transgenic studies and unexpectedly, E3'⁻/-/ mice were found to exhibit no defects in the production of high-affinity Ab when immunized against 2-phenyl-5-oxazolone and these Ab possessed the normal levels of SHM (29). Recognizing that Ag-driven affinity maturation may have selected for survival of this B cell subset, Inlay et al. (28) recently reinvestigated the role of E3' in the process of SHM under physiological conditions without continuous challenge with a single Ag and did find that E3' was necessary to achieve WT levels of SHM in the 5' end of the Igκ gene J-C intron. They further showed that MEi in the native locus played no role in determining the level of SHM. Taking a similar experimental approach to that of Inlay et al. (28), we have found that Ed⁻/-/ mice show about a 2.5-fold decrease in the level of SHM relative to those of WT mice (Fig. 8). This fold decline is in the same range as that seen in E3'⁻/-/ mice (28). We conclude that both E3' and Ed contribute to SHM. Notably, both these enhancers contribute to Igκ gene transcription levels and each also have E2A binding sites, making it difficult to judge which of these defects or both might be responsible for a decline in SHM.

In conclusion, our results together with other published studies on endogenous Igκ locus enhancer knockout mice permit the conclusions that MEi and E3', but not Ed, are required for maximal gene rearrangement (25–27), that E3' and Ed, but not MEi, are required for maximal Igκ rearranged gene transcription (25–28), and that E3' and Ed, but not MEi, are required for maximal SHM in rearranged Igκ genes (28). Because E3' and Ed might have redundant functions to drive Igκ rearranged gene transcription and SHM, one might predict from these combined results that E3' and Ed double knockout mice might be severely impaired for high level expression of rearranged Igκ genes, and totally compromised with respect to the process of SHM.
Disclosures
The authors have no financial conflict of interest.

References