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A Conserved Transcriptional Enhancer Regulates RAG Gene Expression in Developing B Cells

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Summary

Although expression of the RAG1 and RAG2 genes is essential for lymphocyte development, the mechanisms responsible for the lymphoid- and developmental stage-specific regulation of these genes are poorly understood. We have identified a novel, evolutionarily conserved transcriptional enhancer in the RAG locus, called E*rag*, which was essential for the expression of a chromosomal reporter gene driven by either RAG promoter. Targeted deletion of E*rag* in the mouse germline results in a partial block in B cell development associated with deficient V(D)J recombination, whereas T cell development appears unaffected. We found that E2A transcription factors bind to E*rag* in vivo and can transactivate E*rag*-dependent reporter constructs in cotransfected cell lines. These findings lead us to conclude that RAG transcription is regulated by distinct elements in developing B and T cells and that E*rag* is required for optimal levels of RAG expression in early B cell precursors but not in T cells.

Introduction

The variable region exons of immunoglobulin (Ig) and T cell receptor (TCR) genes are assembled from their component V, D, and J gene segments during early B and T cell development by a series of site-specific DNA recombination reactions known as V(D)J recombination (Tonegawa, 1983). Proteins encoded by recombination activating genes 1 and 2 (RAG1 and RAG2) are essential for this process. Expressed at significant levels only in B and T cell progenitors, the RAG proteins are responsible for the recognition and double-stranded (ds) cleavage of conserved DNA elements known as recombination signal sequences (RSSs) that flank all rearranging gene segments (reviewed in Fugmann et al., 2000). Pairs of dsDNA breaks derived from rearranging gene segments are then repaired by the nonhomologous end joining machinery expressed in all cells, resulting in the formation of novel Ig or TCR variable-domain exons. Null mutations in the genes encoding either RAG1 or RAG2 result in an early arrest in lymphocyte development, characterized by the complete absence of V(D)J recombination. Artificial expression of RAG1 and RAG2 is sufficient to activate V(D)J recombination in nonlymphoid cells as assayed by cotransfected rearrangement reporter constructs, indicating that the RAG proteins are the only essential lymphoid-specific components of the V(D)J recombinase.

Normal B and T cell development depends on the regulated timing of gene rearrangement at the various Ig and TCR loci. Regulated transcription of the RAG genes is thought to play a role in this process. In developing B cells, the first wave of RAG expression occurs in pro-B cells in which the Ig heavy chain locus is being rearranged (Li et al., 1993). Productive heavy chain gene rearrangement and pre-B cell receptor (pre-BCR) expression lead to early pre-B cell proliferation and downregulation of RAG expression, a process that may serve to prevent allelic heavy chain gene rearrangement (Grawunder et al., 1995). RAG expression increases again during the small, resting late pre-B cell stage coincident with the second period of V(D)J recombination during which Ig light chain gene rearrangement takes place (Grawunder et al., 1995). Successful light chain rearrangement and subsequent expression of surface IgM result in developmental progression to the immature B cell stage. Surface Ig expression does not necessarily result in the cessation of light chain gene rearrangement, however, and RAG transcripts continue to be expressed in a fraction of normal $sIgM^+sIgD^-$ immature B cells (Grawunder et al., 1995; Li et al., 1993; Rolink et al., 1993). Immature B cells displaying self-specificity continue to transcribe the RAG1 and RAG2 genes and go on to alter their BCR by further light chain gene rearrangement, a process referred to as receptor editing (Pelanda et al., 1997; Radic and Zouali, 1996).

The pattern of RAG expression in developing T cells is very similar to that in developing B cells. RAG transcript levels are high during the early $CD4^-CD8^-$ stages of T cell development and diminish after successful V-to-DJβ rearrangement results in the expression of a pre-TCR and several rounds of rapid proliferation. RAG expression is upregulated again after cell cycle exit, and TCRα locus rearrangement begins (Wilson et al., 1994). Successful TCRα gene rearrangement and surface expression of a complete α/β TCR are insufficient to turn off RAG gene expression. RAG expression and V(D)J recombination continue until TCR engagement during positive selection (Brandle et al., 1992). Positively selected DP thymocytes terminate RAG expression, mature to $CD4^+CD8^-$ or $CD4^-CD8^+$ single-positive (SP) thymocytes, and eventually exit the thymus. Unlike the case in B cells, ongoing TCRα locus receptor editing does not seem to be a prominent mechanism contributing to self-tolerance.

Since regulated expression of RAG1 and RAG2 plays a critical role in the lymphoid and stage specificity of V(D)J recombination, several groups have engaged in

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experiments aimed at identifying the DNA elements and transcription factors that regulate RAG gene expression. The RAG1 and RAG2 genes are physically linked in the genome and are convergently transcribed. Previous work from our laboratory and others has shown that both RAG1 and RAG2 promoters are highly conserved between mouse and human (Brown et al., 1997; Fuller and Storb, 1997; Kurioka et al., 1996; Lauring and Schlissel, 1999; Zarrin et al., 1997). Unlike the murine and human RAG1 promoters that display activity in lymphoid and nonlymphoid cell lines, the murine RAG2 promoter displays lymphoid-specific activity and is regulated differently in B and T cell lines (Lauring and Schlissel, 1999; Wang et al., 2000). Given the complexity of their expression patterns, we and others have speculated that regulated RAG expression requires sequences in addition to promoters such as enhancers, locus control regions (LCRs), or insulators.

To identify potential regulatory elements in the RAG locus, we devised a novel reporter construct in which GFP expression is driven by either RAG1 or RAG2 promoter in conjunction with potential enhancer sequences from the RAG locus in stably transfected cell lines. Using this assay system, we identified a highly conserved RAG locus enhancer. Targeted deletion of this element in mice had a profound effect on B cell, but not T cell, development.

Results

Identification of a New *Cis*-Acting Element within the RAG Locus

In an attempt to identify *cis*-acting elements that contribute to high-level, tissue-, and stage-specific expression of RAG genes, we initially tested restriction fragments generated from an ~120 kb P1 bacteriophage clone of the murine RAG locus for the ability to enhance RAG-2 promoter activity in a transient transfection luciferase assay. These studies led to the identification of a 2.3 kb XbaI fragment (Fragment C in Figure 1A; referred to hereafter as E*rag*) located ~22 kb 5′ of the RAG2 first exon that consistently gave ~2-fold stimulation of promoter activity upon transfection into the Abelson virus transformed pro-B cell line, 220-8 (data not shown). While this enhancement effect was reproducible, its magnitude led us to doubt the biological relevance of E*rag*. To determine whether E*rag* activity might be more striking within the context of chromatin structure, we designed a stable transfection assay of enhancer activity. As depicted in Figure 1B, the chromosomal reporter construct consists of an enhancer test site 5′ of either the murine RAG1 or RAG2 promoter that drives expression of a green fluorescent protein (GFP) cDNA. In addition, the construct contains a neomycin resistance cassette for selection of stable transfectants separated from the reporter cassette by two copies of chicken β-globin insulator (Chung et al., 1993). The insulator should prevent the drug selection cassette itself from influencing reporter construct activity.

The chromosomal reporter construct with or without E*rag* was linearized and then introduced into the 220-8 pro-B cell line by electroporation. Bulk (uncloned) populations of transfectants were selected and expanded in G418. Two weeks after transfection, the levels of GFP expression were determined by flow cytometry. In contrast to our previous results using transient transfection assays in which both the RAG1 (nt −243 to +72) and RAG2 (nt −279 to +123) promoters were active in several pro-B cell lines (Lauring and Schlissel, 1999), "promoter-only" stable transfectants exhibited levels of GFP expression virtually identical to those of untransfected cells (Figure 1C, upper panels). Inclusion of the immunoglobulin heavy chain intronic enhancer (Eμ) in the reporter construct resulted in 10% to 20% GFP$^+$ cells. Control experiments revealed that transfection efficiency was comparable among the various reporter constructs (data not shown). Therefore, the most likely explanation for the lack of GFP expression in the promoter-only constructs is that RAG promoters are unable to overcome the repressive effects of neighboring chromatin structure when integrated into chromosomal DNA.

Interestingly, E*rag*, which showed very modest RAG2 promoter stimulation in the transient transfection assay, had a much greater effect on chromosomal reporter construct activity (30%–40% GFP$^+$ cells) than did the Eμ control (Figure 1C, compare upper and middle panels). This effect was apparent in reporter constructs based on the RAG1 promoter as well (Figure 1C, lower panels). No difference in mean fluorescence intensity of GFP$^+$ cells within transfected populations was observed in Eμ or E*rag*-containing constructs. E*rag* displayed similar RAG enhancer activity in three other RAG-expressing pro-and pre-B cell lines (data not shown). An adjacent 2.3 kb XbaI fragment (Fragment D in Figure1A) as well as a large number of other RAG-locus DNA fragments (Figure 1C, middle panels and data not shown) failed to rescue RAG promoter activity in stably transfected cells, verifying the specificity of the E*rag* effect on the RAG1 and RAG2 promoters. We also found that E*rag* shares with other classic enhancers the ability to function in either orientation (Figure 1C, middle panels).

To determine whether E*rag*-dependent chromosomal reporter construct activity was variegated within individual transfectant clones, we generated 49 single-cell clones from the pool of 220-8 cells stably transfected with the E*rag*-RAG2 reporter shown in Figure 1C. Each clone demonstrated uniform GFP expression levels ranging from none (36%) to ~10-fold above background (10%; see Supplemental Figure S1 at http://www.immunity.com/cgi/content/full/19/1/105/DC1). Thus, we conclude that E*rag* allows the RAG1 or RAG2 promoter to overcome the repressive effects of chromatin structure at only a subset of integration sites.

E*rag* Activates Transcription Specifically in RAG-Expressing B Cell Lines

The results described above demonstrate that E*rag* is capable of activating RAG1 and RAG2 promoter activity in the 220-8 pro-B cell line. We went on to test the ability of E*rag* to regulate the RAG promoters in other B cell, T cell, and nonlymphoid cell lines. Although E*rag* was active in RAG-expressing early B cell lines (Figure 1C and data not shown), it was inactive in mature B cell lines (CH33 and M12) that do not express the endogenous RAG genes (Figure 1D). Failure of E*rag* to activate transcription in mature B cells was not due to unrespon-

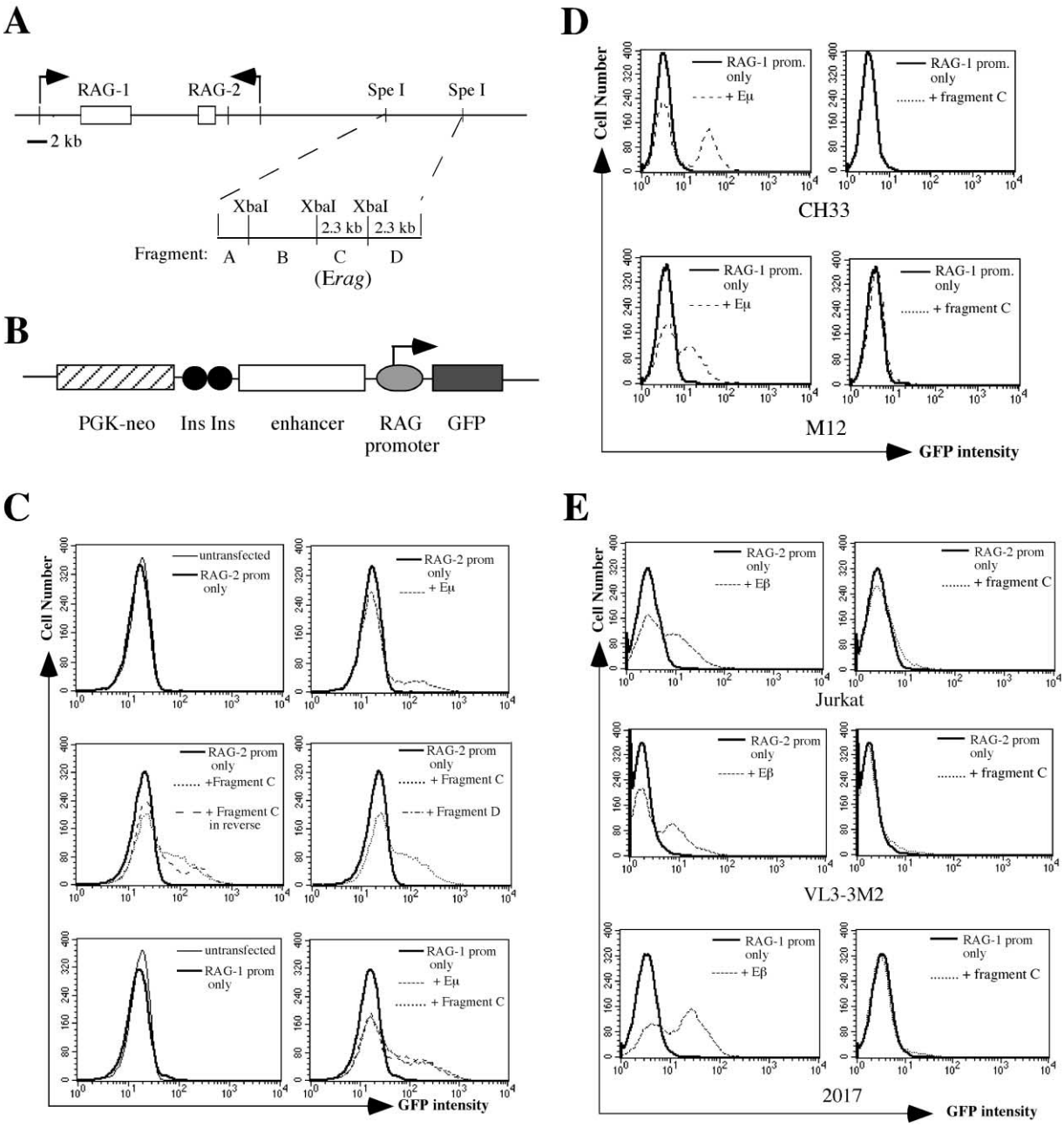

Figure 1. Identification of a RAG Locus Enhancer Using a Stable Transfection Assay

(A) Partial map of the murine RAG locus. The position of a 2.3 kb subfragment (labeled "C" and subsequently referred to in the text as E*rag*) of a 9 kb SpeI fragment beginning 16 kb 5′ of the RAG2 promoter is shown.

(B) Diagram of reporter construct for stable transfection assay. Ins indicates the 1.2 kb chicken β-globin insulator sequence.

(C) FACS histograms of GFP expression in transfected cell lines. The AMuLV-transformed murine pro-B cell line 220-8 was stably transfected with linearized reporter constructs in which a RAG promoter was paired with either Eμ, E*rag* (Fragment C), or Fragment D as indicated. After 2 weeks of selection in G418, pools of surviving cells were analyzed by flow cytometry for GFP expression. The fluorescence histogram of untransfected cells (thin solid line) was overlaid with those of various transfectant pools containing a RAG promoter alone (bold solid line) or in conjunction with either Eμ (positive control), E*rag*, or Fragment D (dashed or dotted lines). Note that histograms of transfected pools from RAG promoter-only constructs and the promoter-plus-Fragment D construct are indistinguishable from that of untransfected cells.

(D and E) Experiments were conducted as described in (C). Recipient cell lines included (D) mature B cell lymphomas CH33 and M12 and (E) T cell lines Jurkat, VL3-3M2, and 2017. Constructs containing Eμ or Eβ served as positive controls in the B cell lines and T cell lines, respectively.

siveness of the RAG2 promoter in these cells since a significant percentage of GFP$^+$cells were observed in both Eμ and SV40 enhancer-containing constructs (Figure 1D and data not shown). Furthermore, inclusion of E*rag* had no effect on RAG promoter activity when the chromosomal reporter construct was introduced into

various RAG-positive or negative T cell lines (Figure 1E), whereas the TCR β enhancer (Eβ) control construct enhanced RAG promoter activity in each T cell line. Taken together, these experiments lead us to conclude that E*rag* is required for mediating developmental stage-specific RAG promoter activity in B cell but not T cell lines.

Striking Conservation of E*rag* among Multiple Mammalian Species

To further test the potential significance of E*rag*, we identified and cloned the fragment from a human RAG locus BAC that crosshybridized to the murine E*rag* DNA fragment. Stable transfection of this 1.7 kb human RAG locus fragment, cloned in the chromosomal reporter construct, into the 220-8 pro-B cell line revealed an increase in the number of GFP^+ cells similar to that found with murine E*rag*, thereby providing functional confirmation that this homologous region contains the human version of E*rag* (see Supplemental Figure S2 at http://www.immunity.com/cgi/content/full/19/1/105/DC1). DNA sequence analysis revealed a 1.1 kb human DNA sequence located in an identical position relative to the RAG2 promoter with greater than 90% sequence identity between the human and murine E*rag* fragments (Figure 2 and data not shown). Using a degenerate PCR approach, we amplified, cloned, and sequenced homologous DNA from the cow, sheep, and pig genomes. As shown in Figure 2, approximately 600–700 bp of E*rag* was highly homologous (~80%) among these five mammalian species. Of particular interest is the sequence of the human-mouse homology region showing several conserved consensus sites for known transcription factors, including E box proteins, Ikaros, LEF/TCF, GATA factors, and Pax-5/BSAP.

Generation of E*rag*-Deficient Mice

To determine the role of E*rag* in the developmental regulation of endogenous RAG locus expression, we used homologous recombination in ES cells to generate mice with a deletion of this element. As shown in Supplemental Figure S3A at http://www.immunity.com/cgi/content/full/19/1/105/DC1, our targeting construct was generated by replacement of a 1.7 kb ScaI-SmaI fragment containing the conserved region of E*rag* with a neomycin resistant gene (neo^r) flanked by loxP sites. Of 50 G418 resistant ES cell clones screened for homologous recombination, five clones were correctly targeted as confirmed by Southern blot analyses (data not shown). Two independent recombinant clones were injected into C57BL/6 blastocysts and the resulting chimeric mice were bred to obtain germline transmission of the targeted allele (designated N). To eliminate the neomycin selection cassette, mice heterozygous for the targeted allele were bred to EIIa-Cre transgenic mice that express Cre recombinase in early embryonic stages. Deletion of the Neo^r gene was confirmed by Southern blot analysis using two flanking probes outside of the targeted region and a Neo probe (see Supplemental Figure S3B at http://www.immunity.com/cgi/content/full/19/1/105/DC1). The heterozygous $Erag^{+/-}$ mice were subsequently crossed to generate $Erag^{+/+}$, $Erag^{+/-}$, and $Erag^{-/-}$ littermates for further analysis.

Deletion of E*rag* Partially Blocks B Cell Differentiation at the Pro-B Stage

We first examined the effect of E*rag* deletion on lymphocyte development by multiparameter flow cytometry. Interestingly, analysis of early B cell populations defined by B220, CD43, and IgM expression showed that the fraction of pro-B cells (IgM^- $B220^+CD43^+$) was increased (from 17.2% to 24.5% of $sIgM^-$ cells), whereas the percentage of pre-B cells (IgM^- $B220^+CD43^-$) was reduced (from 37.5% to 15.6% of $sIgM^-$ cells) in $Erag^{-/-}$ mice compared to wild-type controls (Figure 3A, upper panels). As a result, the pro-B:pre-B ratio in $Erag^{-/-}$ mice is significantly higher than that found in wild-type mice. Furthermore, we observed a marked reduction of IgM^+ cells in $Erag^{-/-}$ bone marrow (from ~29% to ~15% of total mononucleated bone marrow cells; Figure 3A, lower panels).

We found that the absolute numbers of bone marrow cells and splenocytes were decreased in $Erag^{-/-}$ mice, while the number of total thymocytes appeared unaffected (data not shown). Enumeration of different subsets of bone marrow B cells revealed that $Erag^{-/-}$ mice had equivalent numbers of pro-B cells but about 2.5-fold fewer $B220^+$ cells, 3-fold fewer pre-B cells, and 3-fold fewer IgM^+ B cells than wild-type mice (Figure 3C). Similarly, the percentage of $B220^+$ B lymphocytes was also decreased in the spleen compared to wild-type mice. However, in contrast to the marked effect on B cell development, thymocyte development in $Erag^{-/-}$ mice appeared normal, as evidenced by patterns of CD4, CD8, CD44, and CD25 expression that were indistinguishable from wild-type and by equivalent absolute cell numbers (Figure 3B and data not shown). Taken together, these results show that normal B cell but not T cell development depends upon E*rag* activity.

Decreased RAG Transcript Levels in Developing B Cells in $Erag^{-/-}$ Mice

To verify that the partial block at the pro-B and pre-B cell stages of development in $Erag^{-/-}$ mice was due to reduced RAG transcript levels, we designed quantitative real-time RT-PCR assays for RAG1 and RAG2 transcripts. We express the results of these analyses as the ratio of RAG transcript to control HPRT transcript levels. Using these assays, we observed that the expression pattern of RAG transcripts measured by real-time RT-PCR was consistent with previous studies (Grawunder et al., 1995). RAG1 and RAG2 transcripts were high in both pro-B and pre-B cell fractions, somewhat lower in immature B cells, and negligible in mature B cells (Figure 4A).

As shown in Figures 4B and 4C, RAG expression was significantly diminished in $Erag^{-/-}$ B cell progenitors as compared with wild-type littermates in each of three independent sorting experiments. Specifically, deletion of E*rag* resulted in substantial decreases in RAG1 transcript levels (5- to 15-fold), whereas the effect on RAG2 transcript levels was less profound (2- to 3-fold). In agreement with results from the stable transfection studies presented above, these data indicate that E*rag* is critical for normal RAG transcription during B cell development.

Despite the absence of an apparent defect in T cell development, we went on to examine RAG transcript

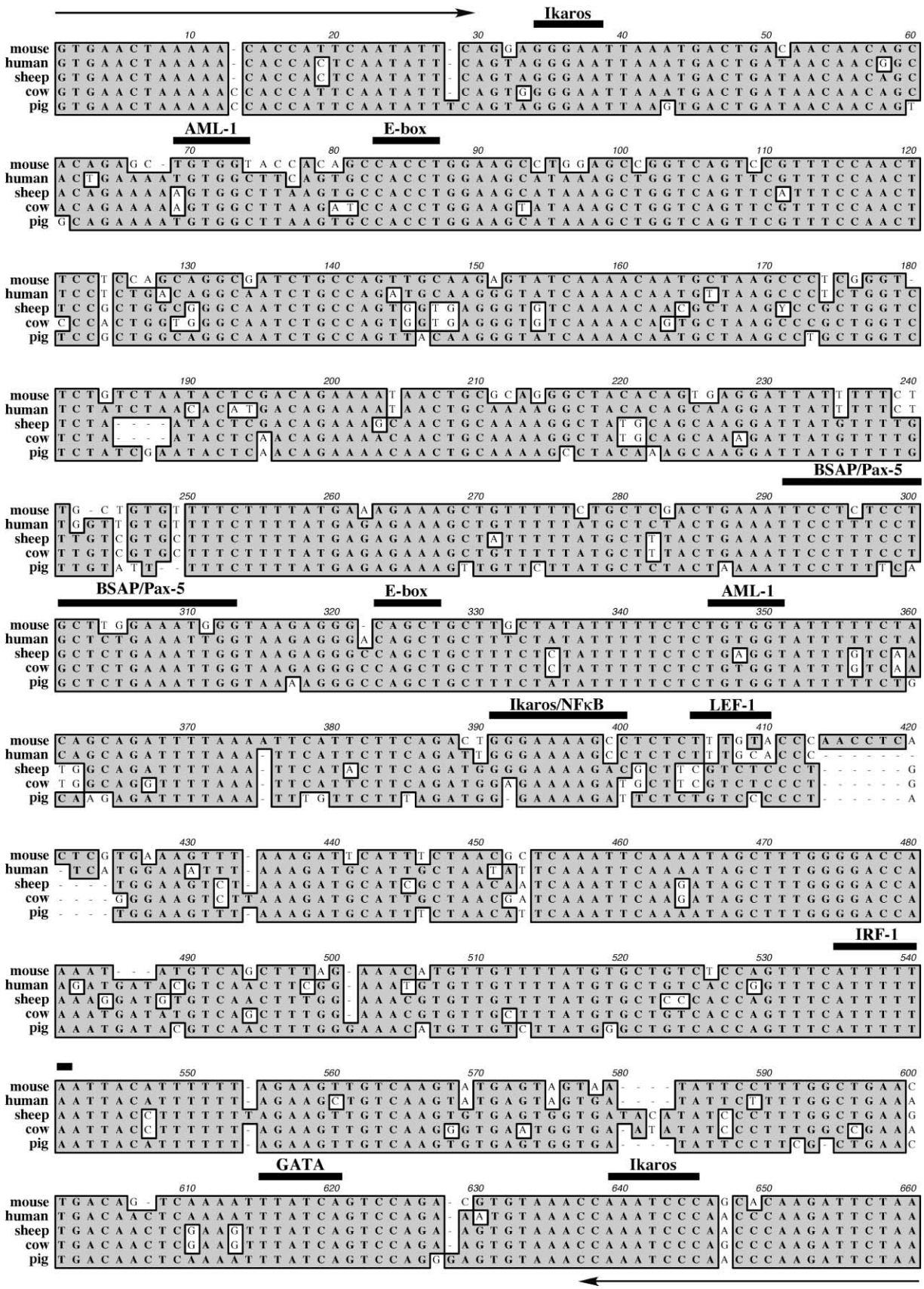

Figure 2. *Erag* Is Highly Conserved among a Set of Five Mammalian Species

ClustalW alignment of *Erag* region sequences from the indicated species. The human sequence was obtained from a 1.7 kb restriction fragment of a human RAG locus BAC identified by hybridization to the murine sequence. Pig, sheep, and cow sequences were obtained from PCR reactions using primers designed based on the human-mouse sequence conservation (positions indicated by arrows). Consensus transcription factor binding sites are indicated in thick solid lines above the DNA sequence.

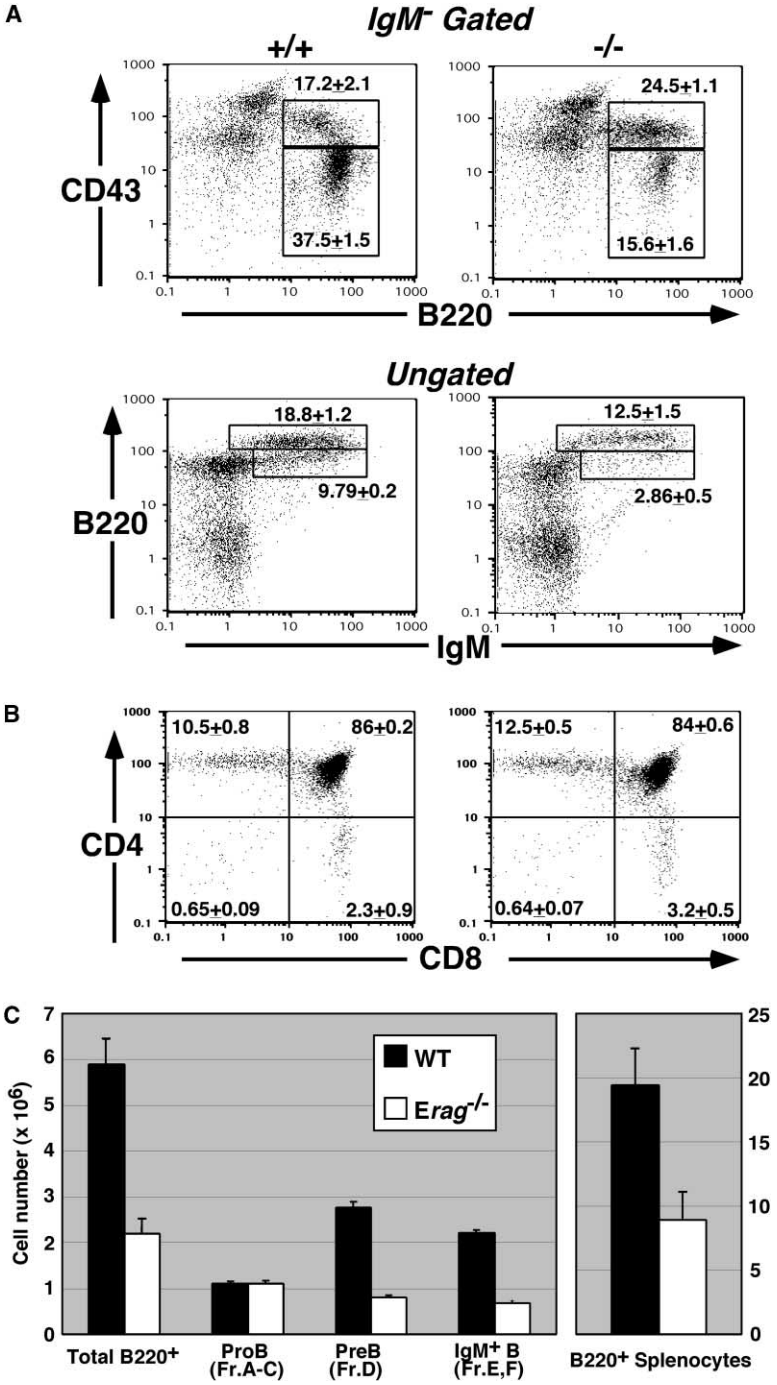

Figure 3. Impaired B Cell Development and Normal T Cell Development in Homozygous E*rag* Mutant Mice

Bone marrow cells, thymocytes, and splenocytes obtained from 6- to 7-week-old wild-type and homozygous mutant mice were analyzed by flow cytometry. The figure shown is representative of staining from at least six separate mice of each genotype. The percentage of cells in each gate is presented as the mean ± SD (n = 6) and is indicated in each gate. All data were gated by forward and side light-scattering prior to analysis. (A) Bone marrow cells from wild-type (left panels) and E$rag^{-/-}$ (right panels) mice were stained with FITC-anti-IgM, PE-anti-B220, and biotin-anti-CD43 revealed by SA-QR. Upper, IgM^--gated bone marrow cells; lower, ungated bone marrow cells. Pro-B cells are $IgM^-B220^+CD43^+$ while pre-B cells are $IgM^-B220^+CD43^-$. (B) Thymocytes (left panel, wild-type; right panel, E$rag^{-/-}$) were stained with FITC-anti-CD8α and PE-anti-CD4 antibodies. (C) Absolute cell numbers were calculated based on the relative percentages determined by FACS analysis in (A) and (B), and total numbers of RBC-free bone marrow cells isolated from both femurs and tibiae of individual mice. Splenic cells from mice of the indicated genotypes were stained with antibody specific for B220, and absolute cell numbers were calculated as above six mice of each genotype were analyzed with the error bars indicating standard deviation of the data.

levels in total thymocytes and in sorted double-negative (DN) or double-positive (DP) thymocytes. Real-time RT-PCR analysis revealed similar RAG1 and RAG2 transcript levels in thymocytes from wild-type mice and E$rag^{-/-}$ mice (Figure 4D). We conclude that deletion of E*rag* has a significant effect on RAG expression in B cell precursors but little or no observable effect on RAG expression in T cell precursors.

V(D)J Recombinase Activity Is Diminished in E$rag^{-/-}$ Mice

To determine the extent to which reduced RAG transcripts levels in developing B cells affected V(D)J recombinase activity, we examined recombination reaction intermediates (dsDNA breaks at recombination signal sequences termed signal broken signal ends or SBE). The levels of these SBEs provide a direct measure of recombinase activity within a population of cells (Constantinescu and Schlissel, 1997; Schlissel et al., 1993). As assessed by LM-PCR, we found that dsDNA breaks at the 5′ of DFL16.1 RSS, which is associated with V_H-to-DJ_H rearrangement, are diminished at least 5-to-10-fold in E$rag^{-/-}$ as compared to wild-type pro-B cell DNA (Figure 5A, 5′D_H SBE). This decrease in V-to-DJ rearrangement affected VH segments all across the locus in a similar fashion (data not shown). To assess the

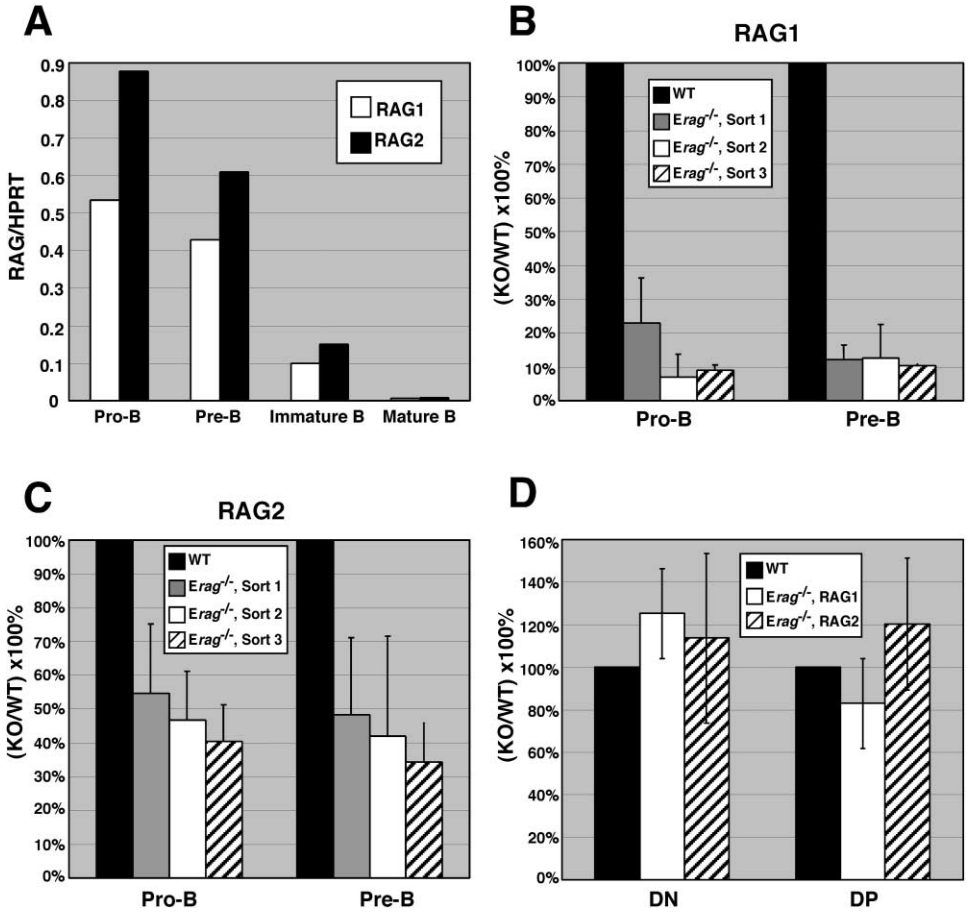

Figure 4. Both RAG1 and RAG2 Transcript Levels Are Reduced in Developing B Cells from E*rag*$^{-/-}$ Mice

Real-time "Taqman" RT-PCR was used to determine the amounts of RAG and HPRT mRNA in FACS-purified pro-B cells (IgM$^-$ B220$^+$CD43$^+$), pre-B cells (IgM$^-$ B220$^+$CD43$^-$), immature B cells (IgM$^+$B220^{lo}IgD$^{-/lo}$), and mature B cells (IgM$^+$B220^{hi}IgD^{hi}).

(A) Relative RAG1 or RAG2 mRNA expression in sorted populations of wild-type bone marrow B cells. RAG1 (open bar) and RAG2 (solid bar) transcripts were normalized to HPRT transcript levels in each sample. Results are in arbitrary units relative to HPRT mRNA.

(B) RAG1 transcript levels in RNA purified from sorted pro-B and pre-B cells from wild-type mice as compared to three individual E*rag*$^{-/-}$ mice are shown. Each assay has been normalized based on HPRT expression and is plotted as a percentage of the wild-type value. Error bars are based on triplicate real-time PCR analyses.

(C) RAG2 transcript levels in wild-type and mutant pro-B and pre-B cells analyzed as in (B).

(D) Relative transcript levels of RAG1 and RAG2 in sorted DN and DP thymocytes. Results represent the mean percentage relative to the wild-type control ± standard deviations for three separate sets of sorts.

extent of D_H-to-J_H rearrangement in pro-B cells, we utilized a pair of primers that amplify a region of germline DNA 5′ of and including J_H1 that is invariably lost upon D_H-to-J_H rearrangement (Hardy et al., 1991). Amplification of genomic DNA from E*rag*$^{-/-}$ pro-B cells showed a significant increase (~9-fold) in retention of the sequence 5′ of J_H1, indicating that D_H-to-J_H rearrangement is less efficient in E*rag*$^{-/-}$ than in wild-type pro-B cells (Figure 5A, top panel).

In addition, we found that dsDNA breaks at the $J_\kappa1$ RSS were decreased at least 10-fold in pre-B cells purified from E*rag*$^{-/-}$ as compared to wild-type mice (Figure 5B). Interestingly, while no obvious differences were detected between mutant and wild-type thymocytes by either flow cytometry or RT-PCR, we observed a subtle decrease (<2-fold) in the levels of DSBs at RSSs 5′ of $D_\beta1$ and various $J_\beta2$ RSSs in total thymocytes from E*rag*$^{-/-}$ mice compared to wild-type mice (Figure 5C). This difference is within the range of variability in the LM-PCR assay, however, and its significance remains uncertain. Thus, decreased levels of RAG1 and RAG2 transcripts in E*rag*$^{-/-}$ mice appear to contribute to a drop in overall levels of RAG-mediated DNA cleavage, resulting in deficient antigen receptor gene rearrangement during B cell but not T cell development.

Induction of RAG Expression In Response to BCR Crosslinking Is Normal in E*rag*$^{-/-}$ Mice

Given the apparently exclusive effect of E*rag* deletion on RAG activity in the B cell lineage, we hypothesized it may be involved in an aspect of RAG regulation thought to occur in B but not T cells—receptor editing. Previous studies from Nemazee and coworkers have defined two populations of immature B cells based on their differential responses to autoantigen stimulation (Melamed et al., 1998). In the presence of autoantigen or anti-BCR antibody, IgM^{lo}/IgD$^-$ cells expressed significantly increased levels of RAG2 mRNA, whereas IgM^{hi}/

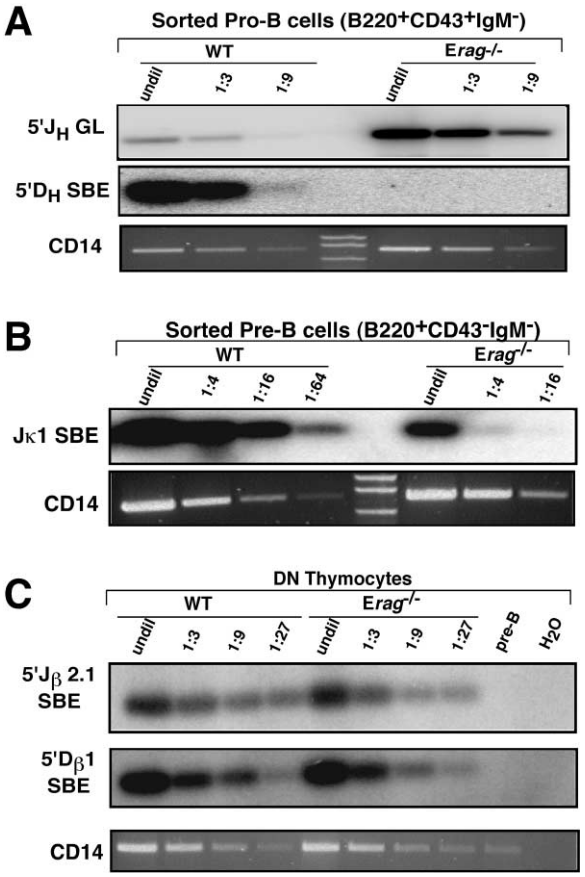

Figure 5. Reduced Levels of V(D)J Recombination in Pro- and Pre-B Cells from E*rag*$^{-/-}$ Mice

Genomic DNA samples purified from sorted populations of B and T cells from wild-type and E*rag*$^{-/-}$ mice were subjected to LM-PCR to detect broken signal-end recombination reaction intermediates (SBE) associated with the D_H (A, middle panel), $J_\kappa 1$ (B), $J_\beta 2.1$ (C, top panel), and $D_\beta 1$ (C, middle panel) gene segments. LM-PCR products were separated on agarose gels, blotted, and hybridized with radiolabeled locus-specific oligonucleotide probes. Serial 3 (A and C)- and 4 (B)-fold dilutions of genomic DNA samples were analyzed in parallel. Direct amplification of a germline DNA sequence 5′ of and including $J_H 1$ was used to quantify the extent of D_H to J_H rearrangement (A, top panel, 5′ J_H GL). Direct PCR amplification of a nonrearranging genomic locus, CD14, was used as a control for DNA abundance in each linker-ligated sample.

IgD$^-$ cells underwent apoptosis and their RAG2 mRNA levels became undetectable. We therefore compared the effects of BCR ligation on the levels of RAG transcripts in IgM^{lo}/IgD$^-$ and IgM^{hi}/IgD$^-$ cells sorted from wild-type and E*rag*$^{-/-}$ BM cultures (Melamed and Nemazee, 1997). While lower relative levels of RAG transcripts were detected in IgM^{lo}/IgD$^-$ B cells of E*rag*$^{-/-}$ mice in the presence or absence of BCR ligation, RAG transcripts increased to a similar extent in response to BCR engagement in both mutant and wild-type cells (10- to 30-fold; Figure 6A). Furthermore, the E*rag* mutation did not alter the striking inactivation of RAG expression observed in IgM^{hi}/IgD$^-$ cells (Figure 6A). Together, these results suggest that E*rag* is not required for the BCR-dependent increases in RAG expression associated with receptor editing. However, we predict that the frequency of receptor editing would be diminished in E*rag*$^{-/-}$ mice because of diminished levels of recombinase present in mutant B lineage cells.

Regulation of RAG1 and RAG2 Gene Expression by Anti-CD3 Crosslinking Is Not Altered in E*rag*$^{-/-}$ Mice

Crosslinking of the TCR-CD3 complex during ex vivo culture of developing thymocytes was previously shown to cause downregulation of RAG expression (Turka et al., 1991). This is thought to mimic the positive selection signal that stops V(D)J recombination in vivo. To test whether E*rag* might play a role in regulating RAG expression in response to TCR signaling, we performed a time-course analysis of RAG mRNA levels in thymocytes following anti-CD3 crosslinking or stimulation with phorbol myristate acetate (PMA) plus ionomycin. RAG1 and RAG2 transcript levels decreased significantly within 2 hr following anti-CD3 crosslinking and in less than an hour following stimulation by PMA plus ionomycin in thymocytes from both wild-type and E*rag*$^{-/-}$ mice (Figure 6B and data not shown). In contrast, treatment of thymocyte cultures with a monoclonal antibody to anti-CD4 or culture in medium without antibodies failed to result in a significant decrease in RAG expression. Thus, we conclude that E*rag* does not play a role in the inactivation of RAG gene expression during T cell development.

E2A Binds to E*rag* in Pro-B Cell Lines and Activates an E*rag*-Dependent Reporter Construct in Nonlymphoid Cells

Computational analysis of the E*rag* sequence revealed six potential E2A binding sites conserved among all five species analyzed (Figure 2 and data not shown). To test whether any of these sites bound E2A in vivo, we performed chromatin immunoprecipitation (ChIP) on material purified from a novel Abelson virus transformed pro-B cell line that expresses a functional FLAG+6xhis-epitope-tagged version of E2A and from thymocytes expressing that same tagged protein (Greenbaum and Zhuang, 2002). As shown in Figure 7A, anti-FLAG immunoprecipitation of chromatin from the tagged (FH) but not the control cell line enriched for E*rag* as well as for sequences from an independently identified distinct RAG locus enhancer (Wei et al., 2002) and the mb-1 promoter, but not the RAG1 promoter. ChIP on thymocytes expressing the epitope-tagged E2A revealed that despite the fact that E*rag* deletion does not effect RAG expression in the thymus, E2A is nonetheless bound to this enhancer in T cell progenitors.

To test whether E2A could activate transcription from these binding sites, we generated a set of luciferase reporter constructs containing either the RAG1 or RAG2 promoter with or without wild-type or mutant E*rag*. The mutant version of E*rag*, termed E*ragm6*, contained point mutations disrupting each of its six potential E2A binding sites. These reporters were transiently cotransfected into the 293T human embryonic kidney cell line in the presence of an empty expression vector or expression vectors encoding either E2A or GATA-3. We found that cotransfection of E2A but not GATA-3 resulted in an E*rag*-dependent stimulation of luciferase activity with wild-type E*rag* but not with E*ragm6* (Figure 7C and data

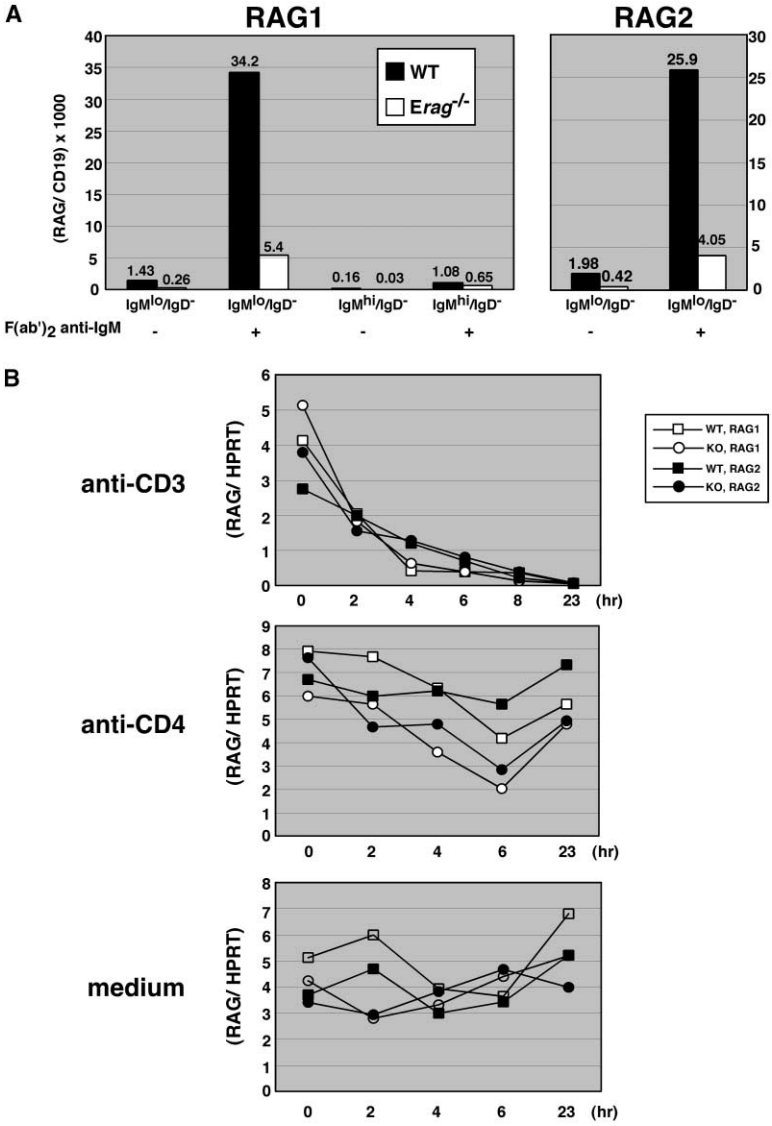

Figure 6. E*rag* Is Not Required for the BCR-Dependent Increases in RAG Expression Associated with Receptor Editing or for the Inactivation of RAG Expression upon TCR Crosslinking

(A) Sorted IgM^{low}/IgD^{-} and IgM ^{high}/IgD^{-} cells from E*rag*$^{-/-}$ and wild-type bone marrow cultures were returned to culture for 48 hr with or without 10 μg/ml F(ab')$_2$ anti-IgM before RNA preparation. RAG1 (left) and RAG2 (right) transcript levels were measured by real-time RT-PCR. The data are expressed as the ratio of RAG transcript to CD19 transcript. Note that amounts of RAG2 mRNA from IgM ^{high}/IgD^{-} cells were not determined because RAG2 transcript levels were below the limits of detection. The numbers indicate actual data values. One of three comparable experiments is shown.

(B) Thymocytes from wild-type (squares) or E*rag*$^{-/-}$ (circles) mice were incubated with medium alone or stimulated with plate-bound anti-CD3 or anti-CD4 antibodies. Cells were harvested and RNA purified at the indicated time points. Real-time RT-PCR was performed to quantify RAG1 (open symbols), RAG2 (filled symbols), and HPRT transcripts. Data shown are the average of duplicates and are representative of two independent experiments.

not shown). These experiments suggest that E2A may be involved in the function of E*rag* in developing B cells.

Discussion

A Stable Transfection Assay for Enhancer Activity

In the present study, we have shown that E*rag*, which lies ~22 kb 5′ of the RAG2 promoter in both the mouse and human genomes, is capable of enhancing expression of a reporter gene from either the RAG1 or RAG2 promoter in RAG-expressing pro- and pre-B cell lines but not in T cell lines. A stable transfection reporter construct was key to the discovery of E*rag* and may be of value in other searches for enhancer activity. Like some enhancers and LCRs, E*rag*'s enhancer activity was demonstrable only when the reporter construct was chromosomally integrated. Given the fact that most previous systematic attempts to identify transcriptional enhancers utilized transient transfection assays, it is possible that elements similar in function to E*rag* may have been missed in these types of studies.

Work from our lab and others identified the RAG1 and RAG2 promoters by virtue of their activity in transient transfection assays. Interestingly, in the present study we found that both RAG promoters are inactive when chromosomally integrated. Our finding that E*rag* was able to increase the frequency of GFP-expressing cells from undetectable to 30%–40% (Figure 1C) suggested that E*rag* may function to prevent the formation of stable repressive structures that silence the RAG promoters. Furthermore, analysis of single cell transfectant clones revealed that RAG promoter activity was either "all-or-none" in the presence of E*rag* (see Supplemental Figure S1 at http://www.immunity.com/cgi/content/full/19/1/105/DC1). We interpret this observation to indicate that E*rag* can overcome the chromatin-dependent inactivation of the RAG promoters, but only at a subset of chromosomal locations.

Additional RAG Transcriptional Control Elements?

Several other groups have engaged in attempts to identify RAG locus regulatory elements. Nussenzweig and

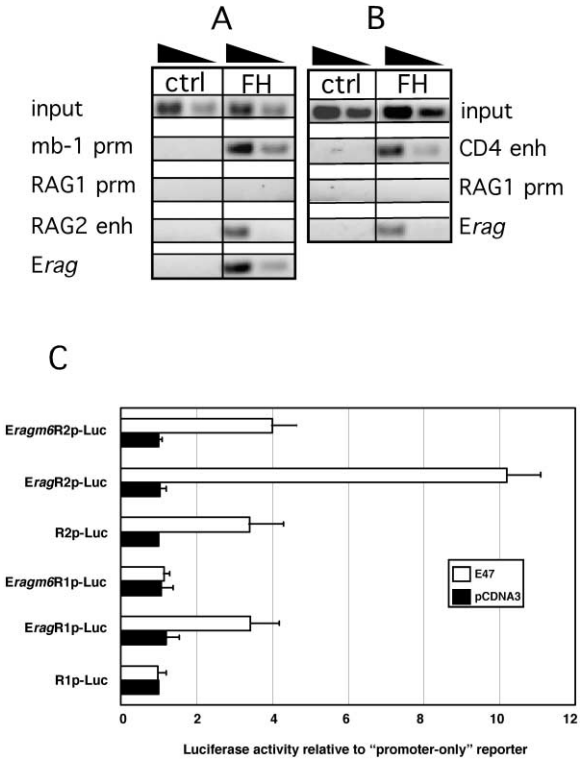

Figure 7. E2A Binds to E*rag* In Vivo within Pro-B Cells and Can Activate an E*rag*-Dependent Reporter Construct in Nonlymphoid Cells

(A) Chromatin immunoprecipitation of E2A-associated DNA sequences. Crosslinked chromatin from either a control pro-B cell line or a pro-B cell line expressing dual-epitope-tagged E2A protein was fractionated using metal-affinity chromatography and anti-FLAG antibody and analyzed by PCR for enrichment of the mb-1 promoter, RAG1 promoter, a previously described RAG locus enhancer, or E*rag*. Input indicates unfractionated chromatin. PCR assays programmed with undiluted and 3-fold-diluted DNA samples are shown. (B) Chromatin immunoprecipitation of E2A-associated DNA sequences from thymocytes expressing dual-epitope-tagged E2A protein performed as in (A). The CD4 enhancer sequence contains a previously studied E2A binding site and serves as a positive control. (C) Transient tranfection luciferase assays. The indicated reporter constructs were transiently transfected along with a pCMV-β-gal control vector and either empty pCDNA or pCDNA-E47. E*ragm6* is a version of E*rag* with point mutations disrupting each of its six potential E2A binding sites. Cells were harvested after 40–48 hr and analyzed for β-galactosidase and luciferase activity. The data, after normalization based on β-gal activity, is plotted as luciferase expression relative to each promoter construct cotransfected with empty pCDNA (black bars). The error bars indicate standard deviation of three independent experiments.

colleagues, using modified bacterial artificial chromosome (BAC) clones of the RAG locus as transgenic reporter constructs, demonstrated that at least two distinct regulatory elements 5′ of the RAG2 promoter appear to be required for proper expression of RAG1 and RAG2 in the B and T cell lineages (Yu et al., 1999). One such element, which could activate reporter expression in B cell progenitors as well as $CD4^{-}CD8^{-}$ double-negative (DN) T cell precursors, was localized to a 10 kb region immediately 5′ of the RAG2 promoter. A second element necessary for proper RAG expression in DP thymocytes was found to lie between 30 kb and 70 kb 5′ of the RAG2 promoter. A second group independently found a DNase I hypersensitive site ~8 kb 5′ of the RAG2 promoter. DNA sequences in the vicinity of this hypersensitive site had B lineage and DN T cell enhancer activity in transgenic mice (Wei et al., 2002), consistent with the BAC transgenic studies noted above. Finally, a third group (Monroe et al., 1999), using transfected $RAG2^{-/-}$ ES cells and the $RAG2^{-/-}$ blastocyst complementation assay, reported that a genomic fragment containing the RAG2 promoter region and 9 kb of 5′ sequence is sufficient to rescue both B and T cell development, suggesting that 9 kb of sequence upstream of the RAG2 promoter has adequate information to direct RAG2 expression in both B and T lineages.

None of these studies revealed the existence of a *cis*-regulatory element within sequences between 10 kb to 30 kb 5′ of the RAG2 promoter. In the case of the BAC transgene reporters in particular, the region encoding E*rag* was fully dispensable for B cell-specific RAG expression (Yu et al., 1999). This is surprising given the striking effect of E*rag* deletion reported in the present study. It is possible, however, that despite their large size, BAC transgenic reporter constructs are subject to position effects. Furthermore, these large reporters may still be missing sequences from within the locus that are necessary to see the effects of E*rag*. It is also possible that the BAC reporter transgenes integrate into the chromosome as tandem multicopy arrays and that this type of chromosomal structure obviates the requirement for E*rag* activity. Our cell line studies suggest that E*rag* may function, at least in part, by overcoming the repressive effects of chromatin structure. Tandem arrays of transgenes might be less subject to this sort of chromatin effect than a single copy genetic locus. Finally, the half-life of the GFP reporter protein in developing lymphocytes may be such that it is an insensitive indicator of changes in RAG locus transcript levels.

While the deletion of E*rag* results in a partial block in B cell development, RAG mRNA expression was not completely abolished. It would be of interest to test whether E*rag* and the previously identified DNase I hypersensitive site enhancer element (Wei et al., 2002) might cooperate to result in stronger B cell-specific enhancement of RAG promoter activity in the stable transfection assay. Several sets of experiments have revealed that subset- and stage-specific expression of the CD4 and CD8α/β genes is achieved by combined actions of different *cis*-elements (Ellmeier et al., 1999). A similar situation exists in the murine TCRγ locus where two enhancer elements separated by many kilobases of DNA are both required for appropriate TCRγ locus transcription (Xiong et al., 2002). Thus, it is likely that a set of elements is involved in generating the observed pattern of transcriptional regulation of RAG gene expression in developing B and T cells.

Mechanisms of Action of E*rag*

Since activation of transcription by E*rag* was very inefficient when assayed by transient as compared to stable transfection in pro-B cell lines, we conclude that chromosomal structure is involved in the mechanism of E*rag* function. In this context, we hypothesize that E*rag* may serve as a "platform" to recruit general chromatin re-

modeling factors. Our comparison of the E*rag* sequence among a group of five mammalian species revealed several conserved transcription factor binding sites. Of particular interest are binding sites for E2A, Pax5/BSAP, GATA, Ikaros, and LEF-1. Mutations in the genes encoding each of these factors have profound effects on early lymphoid development (reviewed in Henderson and Calame, 1998).

We found that E2A but not GATA-3 expression could activate either RAG promoter in an E*rag*-dependent fashion in nonlymphoid 293T cells and that mutation of the E2A binding sites within E*rag* abrogated this effect (Figure 7C). This, combined with ChIP data showing that E2A is bound to E*rag* in transformed pro-B cells (Figure 7A), strongly implicates E2A in the mechanism of E*rag* function. Interestingly, we found that E2A is bound to E*rag* in thymocytes (Figure 7B) despite the fact that RAG expression is normal in E*rag*$^{-/-}$ thymus. This observation suggests that E*rag* likely requires factors in addition to E2A for full activity or that even when active, E*rag* is not limiting for RAG expression in T lineage cells. Further in vivo footprinting, ChIP, and mutational analyses will be required to define the minimal E*rag* sequence and to determine which other transcription factors contribute to the lineage-restricted activity of E*rag*.

Experimental Procedures

Cell Culture and Stable Transfection Reporter Assay

The reporter construct shown in Figure 1B has been previously described (Wang et al., 2000). The 2.3 kb murine XbaI enhancer fragment (Fragment C in Figure 1A) was cloned in both orientations into a polylinker site upstream of either the RAG1 promoter (from −243 to +72, [Brown et al., 1997]) or RAG2 promoter (from −279 to +123 [Lauring and Schlissel, 1999]). A 1 kb XbaI fragment containing the Eμ enhancer and a 4 kb HindIII fragment containing the Eβ enhancer were used as positive controls. Conditions for growth, electorporation, and selection of stable transfectants of all cell lines were described previously (Lauring and Schlissel, 1999). For single cell cloning, individual G418-resistant clones were generated by limiting dilution of previously generated resistant pools.

Targeted Deletion of E*rag*

See Supplemental Data at http://www.immunity.com/cgi/content/full/19/1/105/DC1.

B Cell Culture

B cell precursors from mutant and wild-type mice were grown in vitro as previously described (Melamed and Nemazee, 1997). In brief, BM cells were depleted of erythrocytes using buffered NH_4Cl solution and filtered through sterile nylon mesh to removed bone fragments and debris. Cells were cultured at 2×10^6 cells/ml for 5 days with IMDM (GIBCO) supplemented with 10% FCS (a gift from Dr. D. Namazee) and 50-100 U/ml of IL-7, derived from the culture supernatant of J558L cells transfected with the murine IL-7 cDNA. For induction of differentiation, cells were harvested, washed three times in medium without IL-7, and cultured on a semiconfluent layer of γ-irradiated S17 stromal cells for 24 to 36 hr in medium without IL-7. At day 6 or day 7 of cultures, B cell precursors were harvested and then stained with FITC-anti-IgM and PE-anti-IgD. IgM$^+$IgD$^-$ immature B cells were sorted into IgM^{low}IgD$^-$ and IgM^{high}IgD$^-$ cell populations. Sorted cells were washed and recultured for 48 hr on monolayers of irradiated S17 in the absence or presence of $(Fab')_2$ anti-mouse IgM (Jackson ImmunoResearch).

Flow Cytometry and Sorting

Single-cell suspensions from BM (two femurs), thymus, and spleen were prepared and stained with fluorochrome (FITC, PE, or Cychrome)- or biotin-conjugated (bi) monoclonal antibodies as previously described (Dillon et al., 2000). The following antibodies were obtained from BD PharMingen (San Diego, CA): FITC-anti-IgM$^{a/b}$ (II/4), -CD4 (RM4-5); PE-anti-B220 (RA3-6B2), -IgD (11-26), -CD4 (RM4-5), -CD25 (PC61), -CD8 (53-6.7); Cyc-anti-CD44 (IM7), -B220 (RA3-6B2). Bi-anti-CD43 (S7) was purified in our laboratory. The remaining antibodies were purchased from Caltag (San Francisco, CA): bi-anti-CD3ε (500A2), -CD4 (CT-CD4), -CD8α (CD-CD8a). PE-streptavidin was obtained from Sigma. FACS analysis was performed on an Elite XL flow cytometer (Beckman Coulter) and data were analyzed with Flowjo (Tree Star, Inc.).

For sorting of pro-/pre-B cells, BM B cells from mutant and wild-type mice were enriched by negative selection using rat anti-mouse IgM MicroBeads (Miltenyi Biotech), followed by positive selection using CD19 MicroBeads. Cells were then stained with anti-B220 and anti-CD43 and sorted into B220$^+$CD43$^+$ and B220$^+$CD43$^-$ fractions. DN thymocytes were isolated by staining thymocytes with bi-anti-CD3ε, bi-anti-CD4, and bi-anti-CD8 followed by negative selection using SA-MicroBeads. DP thymocytes were purified from mutant and wild-type thymocytes stained with PE-anti-CD4 and FITC-anti-CD8α by use of Epic Elite flow cytometer (Beckman Coulter). Purity of all of the sorted cells was 93%–97% as determined by re-analysis.

Real-Time RT-PCR Analysis of RAG mRNA Expression

Total RNA from sorted cells was isolated using Trizol (Invitogen), primed with random hexamers, and reverse-transcribed with SuperScript II (Invitrogen) or Omniscript (QIAGEN) according to the manufacturer's protocols. Real-time PCR analysis utilized an ABI Prism 5700 Sequence Detection System (Applied Biosystems) and was carried out in accord with the manufacturer's instructions. In most cases, data were represented as transcript abundance relative to HPRT, except the experiment shown in Figure 6, in which RAG transcripts were normalized to CD19 abundance. Real-time PCR was carried out in duplicate or triplicate at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Data collected were analyzed using sequence detection software supplied with the instrument and plotted using Microsoft Excel. The following primers and probes were used. RAG1 sense 5′-CATTCTAGCACTCTGGC CGG, RAG1 anti-sense 5′-TCATCGGGTGCAGAACTGAA, RAG1 probe FAM-5′-AAGGTAGCTTAGCCAACATGGCTGCCTC-3′-Tamra, RAG2 sense 5′-TTAATTCCTGGCTTGGCCG, RAG2 anti-sense 5′-TTC CTGCTTGTGGATGTGAAAT, RAG2 probe FAM-5′-AGGGATAAGCA GCCCCTCTGGCC-3′-Tamra, CD19 sense 5′-AATCCACGCATTCAA GTCCAG, CD19 anti-sense 5′-GAGCCCTCCTCGCTGTCTG, CD19 probe FAM-5′-CTTCTTCCAGTCCTGTTTCATGGCTCTGAG-3′-Tamra. For the quantitation of HPRT, the PCR reactions were done as previously described (Grogan et al., 2001).

LM-PCR

Genomic DNA was isolated and LM-PCR performed as described previously (Constantinescu and Schlissel, 1997; Schlissel et al., 1993; Stanhope-Baker et al., 1996). LM-PCR products were separated on a 2% agarose gel, transferred to a nylon membrane (Hybond XL, Amersham) under alkaline conditions, and hybridized with ^{32}P-labeled locus-specific internal oligonucleotide probes as previously described. Approximately 800 bp of germline sequence extending from 5′ of J_H1 to 3′ of J_H2 was PCR amplified from pro-B cell genomic DNA with the primers μ^0 and J_HB3 as described previously (Constantinescu and Schlissel, 1997; Schlissel, 1998). The products of control CD14 amplification (typically 26 cycles) were detected by ethidium bromide staining of 2% agarose gel. Primers for CD14 amplification were previously described (Schlissel et al., 1993).

T Cell Activation

T cell stimulation was performed as previously described (Turka et al., 1991). In brief, unfractionated thymocytes were freshly isolated from E*rag*$^{-/-}$ mice and wild-type littermates (3–4 weeks old). 5×10^5 or 10^6 cells were incubated in duplicate with plate-bound anti-CD3 (145-2C11, a gift from Dr. J. Allison, coated 5 μg/ml), or anti-CD4 (RM4-5, BD Pharmingen, coated 5 μg/ml) or medium alone for the indicated times.

Transient Transfection Assay

All mutations in six E2A binding sites were made using the Quickchange mutagenesis kit (Stratagene). The sequences of mutagenic primers used for simultaneous mutagenesis were as follows:

5′-GGTACCACAGC**ag**CCTG**G**AAGCCTGGAGCCGGTCAG-3′,
5′-GGCGATCTGC**CAGTgc**CAAGAGTATCAAAACAATGCTAAGC-3′,
5′-GGGTAAGAGGGC**CAGCgc**CTTGCTATATTTTTCTCTGTGG-3′,
5′-AAAGACTCCT**CAGAgc**AGAAG**gcTGTG**GCTTTGAACAAGT
AAC-3′,
5′-CGCTCTCCTGTCAGT**gcTCTG**CTCCTCAGTGCTCTCTG-3′

The E box sequences were shown in bold, with lowercase letters representing nucleotide substitutions. The 2.3 kb wild-type or mutant versions of murine E*rag* fragment were cloned into a polylinker site 5′of either the RAG1 (−243/+72) or RAG2 promoter (−279/+123) from the pR1p/R2p-Luc promoter-only constructs described previously (Lauring and Schlissel, 1999). 2 μg of each luciferase reporter construct together with 250 ng of pCMV-β-gal and 1 μg of either empty vector (pCDNA3) or expression plasmids coding for either E47 (pCDNA-E47) or GATA-3 (pCDNA-GATA-3) were transfected into 293T cells using Polyfect (Qiagen) according to the manufacturer's instructions. Cells were harvested 40–48 hr later and assayed for both luciferase and β-galactosidase activities as described (Lauring and Schlissel, 1999). Transfection efficiency was normalized using β-galactosidase activity and measured relative to a promoterless control luciferase construct. Each assay was performed in triplicate.

Chromatin Immunoprecipitation

Fixed, soluble chromatin was prepared and analyzed essentially as described from a wild-type Abelson virus transformed pro-B cell line and a mutant cell line expressing a FLAG and 6xhis-tagged E2A gene or from thymocytes isolated from a FLAG and 6xhis-tagged E2A mutant mouse (Greenbaum and Zhuang, 2002). Crosslinked E2A-bound DNA fragments were purified by sequential Talon metal affinity resin (Clontech) and anti-FLAG epitope immunoaffinity chromatography. A series of 3-fold dilutions of input chromatin and immunoprecipitated DNA from $E2A^{FH}$ and $E2A^{GFP}$ control cell lines or thymocytes were PCR amplified for 38 cycles (94°C 1 min; 57°C 1 min; 72°C 1 min with 2 min final extension at 72°C) in a 20 μl PCR reaction containing 3 mM $MgCl_2$ and *Platinum Taq* polymerase (Invitrogen). Each entire PCR sample was then resolved on a 1% agarose gel and visualized by ethidium bromide staining. Oligonucleotide primers (IDT DNA) used in PCR for E*rag* detection were: *ERAG1* (5′- TATTCAGGAGGGAATTAAATGAC-3′) and *ERAG2* (5′-GAC AGAACCCGAGGGCTTAGCAT-3′). Primers for the *RAG1* promoter, *RAG2* enhancer (Wei et al., 2002), and *mb-1* promoter were described previously (Greenbaum and Zhuang, 2002).

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SUPPLEMENTAL DATA

Summary of this Article
Full Text of this Article

Supplemental Data for:

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Supplemental Figure S1. Analysis of Single Cell Clones Reveals a Unimodal Distribution of Fluorescence in Each Member of a Transfected Cell Pool

Single-cell clones were derived by limiting dilution from a pool of 220-8 cells stably transfected with the reporter construct containing the RAG2 promoter and E*rag*. Forty-nine such clones were individually assayed by flow cytometry for GFP expression. (A) Representative FACS histograms of several individual E*rag*-containing clones (broken lines) are shown together with fluorescence of "promoter-only" transfectants (broken lines). (B) Graph of the distribution of mean fluorescence intensities of all of the individual clones analyzed.

Supplemental Figure S2. The Human DNA Sequence Homologous to Murine E*rag* Displays Enhancer Activity

A 1.7 kb homologous human restriction fragment was cloned into the RAG2 promoter GFP reporter construct and tested for enhancer activity in the stable transfection assay in 220-8 cells. A fluorescence histogram of a pool of stable transfectants is shown (dashed line) along with a pool of promoter-only control transfectants.

Supplemental Figure S3. Generation of E$rag^{-/-}$ Mice

(A) Schematic diagrams and partial restriction maps of the endogenous murine E*rag* genomic region (top), the targeting vector (upper middle), and targeted allele before (N allele) and after (allele) Cre-mediated deletion of the Neo^r gene (lower middle and bottom). A 1.7 kb ScaI-SmaI portion of the E*rag* region that is highly conserved between mouse and human sequences was replaced with the loxP (triangle)-flanked neo^r gene. Thick solid lines indicate homology arms. The shaded region within E*rag* represents ~1 kb homologous region shown in Figure 3. A diphtheria toxin expression cassette (DT) was included outside of the region of homology to allow selection against randomly integrated plasmid. The bars represent the 5 and 3 probes used for analysis of homologous recombination. The expected sizes of restriction fragments detected by 5 and 3 probes are shown. Arrowheads show positions of PCR primers used to screen for

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deletion of neo gene (primer a and c), and presence of endogenous allele (primer a and b). Asterisks represent restriction sites. RI, EcoRI; H, HindIII; Sc, ScaI; Sm, SmaI; Sp, SpeI; and XbaI, X.

(B) Southern blot analysis of tail DNA from wild-type (+/+), and various mutant mice (heterozygous N allele mutant (N/+), heterozygous allele mutant (-/+), and homozygous allele mutant -/-). Tail DNA samples were analyzed by digestion with HindIII (left) or EcorRI (right) and Southern bolts were hybridized to 5 or 3 probes, respectively. HindIII fragments derived from wild-type, N and alleles are 5 kb, 8 kb, and 6.8 kb, respectively. EcoRI digestion of genomic DNA yields restriction fragments of 8 or 3 kb corresponding to wild type or targeted alleles, respectively. Elimination of neo^r gene was verified by Southern blot analysis using a probe containing neo^r gene (bottom, left panel).

Supplemental Experimental Procedures

Gene Targeting and Generation of E*rag*$^{-/-}$ Mice

A 9 kb SpeI-SpeI fragment from the P1 phage genomic clone containing the murine RAG locus was subcloned into pBluescript II (Stratagene) to generate pBSK-SpeI-SpeI. A 3.6 kb 5 homologous arm (EcoRI-ScaI fragment) and 2.3 kb 3 homologous arm (SmaI-SpeI fragment) were subcloned to XhoI and HindIII sites in pKO915 (Stratagene), respectively, and both insertion sites were destroyed in the process of cloning. Subsequently, diphteria toxin chain gene cassette (DT) from the pKOSelectDT (Lexicon Genetics Inc.) was inserted into the RsrII site. As a selection marker, the loxP-flanked neomycin resistance gene from pLZNeo (Ferradini et al., 1996, a gift from Dr. Hua Gu) was cloned into the unique BamHI site between two homologous arms (see Supplementary Figure S3A).

The targeting vector was transfected into TC1 ES cells (Deng et al., 1996; Monroe et al., 1996b) by electroporation, and cells were selected in medium containing G418. G418-resistant clones were screened for homologous recombination by PCR and confirmed by genomic Southern analysis of HindIII or EcoRI-digested DNA, probed with 5 or 3 probe (see Supplementary Figure S3A), respectively. Two independent ES clones were injected into C57BL/6 blastocysts. Chimeric male mice were initially bred to C57BL/6 mice and germ-line transmitters were identified by coat color and confirmed by HindIII digest of tail DNA. To delete the floxed-*neo*^{r} cassette, offspring were bred to EIIa-cre transgenic mice (Gorman et al., 1996; Lakso et al., 1996) and tail DNA was screened by digestion with HindIII and hybridization with a 5 probe. Deletion of the neomycin resistance gene results in a change in the size of the hybridizing allele from 8 kb to 6.8 kb. In order to avoid any debilitating effect from the Cre transgene (Silver and Livingston, 2001), several additional crosses between heterozygous mice (/+) were done and screened for the absence of the Cre transgenes. Subsequently, the Cre negative heterozygous mice were mated to generate littermates with wild-type (+/+), heterozygous (+/-), and homozygous mutant (/) genotypes for analysis. All mice analyzed in this study were at the age of 6–7 weeks (Figure 3–5) and 8–10 weeks (Figure 6). The following primers were used for genotyping (indicated in Supplementary Figure S3A). Primer a: 5-TAGTGCTACAAACGCAGGAGGATTC-3. Primer b: 5-GTGCTTGAAACTGAACCCA-3. Primer c: 5-GGGTGAAATAGGGGTATGCTGAG-3

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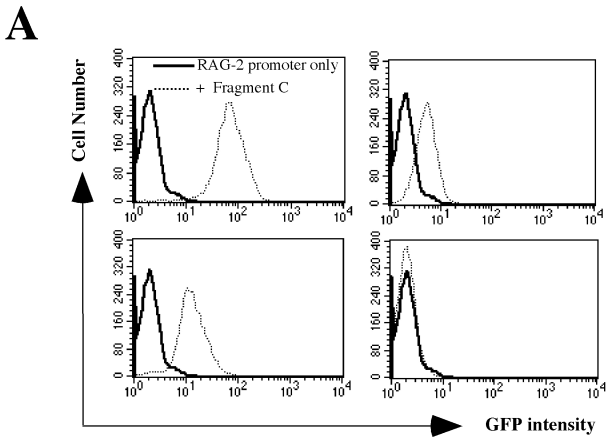
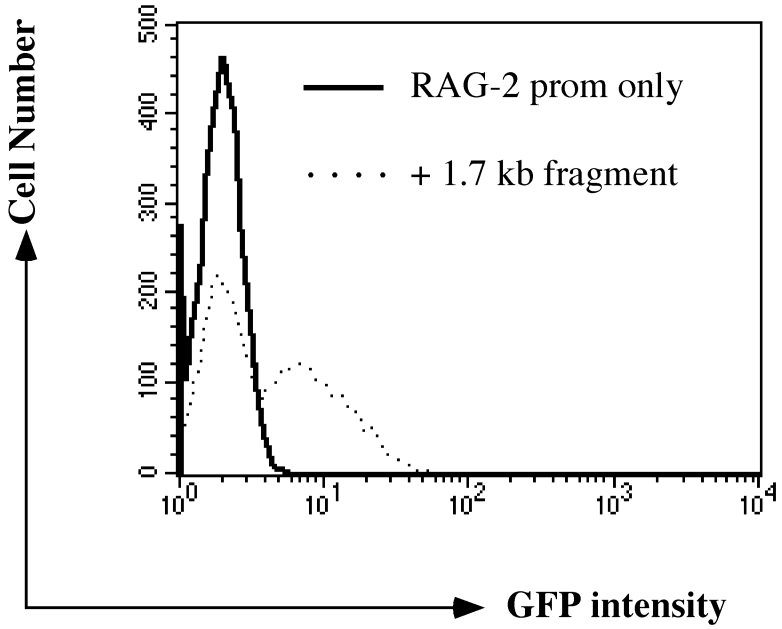
Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3