

B Lineage-specific Regulation of V(D)J Recombinase Activity Is Established in Common Lymphoid Progenitors

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Abstract

Expression of V(D)J recombinase activity in developing lymphocytes is absolutely required for initiation of V(D)J recombination at antigen receptor loci. However, little is known about when during hematopoietic development the V(D)J recombinase is first active, nor is it known what elements activate the recombinase in multipotent hematopoietic progenitors. Using mice that express a fluorescent transgenic V(D)J recombination reporter, we show that the V(D)J recombinase is active as early as common lymphoid progenitors (CLPs) but not in the upstream progenitors that retain myeloid lineage potential. Evidence of this recombinase activity is detectable in all four progeny lineages (B, T, and NK, and DC), and *rag2* levels are the highest in progenitor subsets immediately downstream of the CLP. By single cell PCR, we demonstrate that V(D)J rearrangements are detectable at IgH loci in ~5% of splenic natural killer cells. Finally, we show that recombinase activity in CLPs is largely controlled by the *Erag* enhancer. As activity of the *Erag* enhancer is restricted to the B cell lineage, this provides the first molecular evidence for establishment of a lineage-specific transcription program in multipotent progenitors.

Key words: B lymphopoiesis • V(D)J recombination • lineage restriction • hematopoiesis • stem cell • transcription

Introduction

Commitment of hematopoietic progenitors to the B or T lineage is associated with the production of transcription factors that initiate expression of proteins essential to the B or T cell fate and repress proteins involved in alternative fates (1, 2). The V(D)J recombinase is an enzyme complex that is absolutely required for normal B and T cell production (3, 4). In the absence of recombinase activity, lymphoid development is abrogated at an early stage resulting in severe combined immune deficiency. Therefore, characterizing developmental regulation of V(D)J recombinase activity in multipotential hematopoietic progenitors is fundamental to understanding progression to the B and T cell lineages.

The V(D)J recombinase is composed of recombinase-activating gene (RAG)1 and RAG2 as well as the DNA repair proteins Ku70 and Ku80, DNA-PKcs, DNA ligase IV, XRCC4, and Artemis (5–14). RAG1 and RAG2 are tightly regulated: *rag* expression is limited to specific developmental subsets of B and T lymphocytes (10, 11, 15–17), and RAG2 protein must be resynthesized and relocated to the nucleus with every cell cycle (18–20). Like RAG2, nonhomologous end joining activity may also be regulated by phosphorylation (21). Thus, *rag* transcription, although required for recombinase function, is not necessarily a definitive indicator of recombinase activity (15, 22, 23).

rag expression is differently regulated in B versus T lineage progenitors, and the cis-acting elements that control both development and lineage specificity are just beginning to

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Abbreviations used in this paper: CJ, coding joint; CLP, common lymphoid progenitor; ETP, early thymic T lineage progenitor; RAG, recombinase-activating gene; RSS, recombination signal sequence; SJ, signal joint.

be resolved. The 10-kb region upstream of *rag2* is sufficient for both complementing lymphocyte development in *rag2*^{-/-} animals (24) and controlling normal expression of *rag1* and *rag2* in B and T lineage progenitors (17). At least two enhancers have been identified within this locus, one at ~8 kb and a second at ~22 kb upstream of *rag2* (25, 26). The latter of these, identified as *Erag*, is particularly interesting because it exhibits B lineage specificity. Mice lacking *Erag* have severely reduced expression of *rag1* and *rag2* in B lineage progenitors leading to a partial block in B cell development, whereas T cell development remains unaffected.

Both B and T lineage progenitors can be derived from the same multipotent CLP (27, 28), and it remains unknown whether *Erag* begins to exert influence only in cells committed to the B lineage. Interestingly, even though bone marrow CLPs retain the ability to produce both B and T cells, these multipotential progenitors may not contribute to each lineage equally. Specifically, *ikaros* knockout

mice lack CLPs but are nonetheless able to generate near normal frequencies of early thymic T lineage progenitors (ETPs) (29). These and additional data suggest that CLPs are early B lineage progenitors, whereas ETPs are early T lineage progenitors. However, the molecular basis underlying this observation remains unknown.

In addition to T and B cells, CLPs can give rise to NK cells and DCs in vivo. The close developmental relationship between these cells is further supported by observations that *lin*⁻*ckit*^{lo} progenitors can produce either CD19⁺ B cells or DX5⁺ NK cells (30), B lineage precursors share developmental markers with DC precursors (28), and CD19⁺ B lymphocytes cultured in vitro can give rise to DEC205⁺CD11c⁺ cells (31). Despite these developmental relationships and the fact that IgH recombination events are detectable in CLPs (29), recombinase expression is widely considered to be restricted to lineage-committed B and T progenitors.

To investigate expression and control of recombinase activity in multipotential hematopoietic progenitors and their

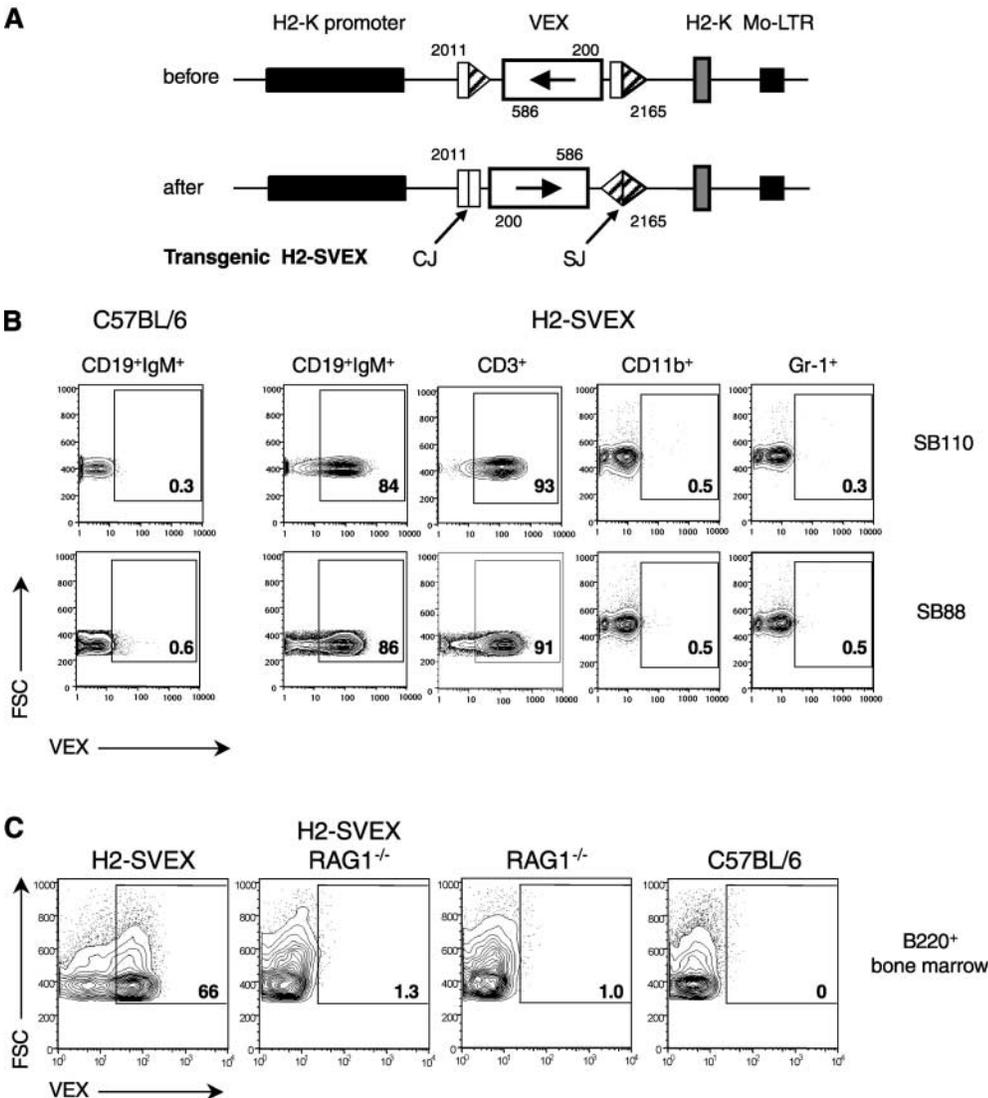


Figure 1. (A) The transgenic H2-SVEX substrate contains VEX (white rectangle) driven by the murine H2K promoter (black rectangle). VEX within the substrate is initially in the antisense orientation and is flanked by V(D)J recombination signal sequences (triangles) which direct inversional recombination. Primers used to discriminate H2-SVEX before and after rearrangement are indicated (2011, 200, 586, and 2165; as described in Materials and Methods). (B) Splenocytes from SB110 and SB88 H2-SVEX animals were stained with antibodies to detect CD19⁺ IgM⁺ B cells, CD3⁺ T cells, CD11b⁺CD3⁻CD19⁻NK1.1⁻ myeloid cells, or Gr-1⁺ CD19⁻CD3⁻NK1.1⁻ granulocytes and subsequently examined for VEX expression. The percentage of VEX⁺ cells in the gate is given, and outliers are shown. (C) H2-SVEX recombination depends on RAG1. B220⁺ B cells in the bone marrow were examined for VEX expression in H2-SVEX SB110, H2-SVEX RAG1^{-/-}, RAG1^{-/-}, and non-transgenic C57BL/6 control mice. The percentage of VEX⁺ cells in the gate is given. H2-SVEX RAG1^{-/-} mice were identified by PCR analysis of the SVEX cassette as depicted in A and Fig. 2 A. Identical results were obtained with SB88 H2-SVEX RAG1^{-/-} mice (not depicted). The data presented are representative of six independent experiments.

immediate downstream progeny *in vivo*, we developed a flow cytometric assay in which V(D)J recombination of a transgenic substrate is indicated by VEX-GFP fluorescence. Within specific developmental subsets of lymphocytes, we can distinguish individual cells that begin to express the recombinase. This approach, which has not previously been possible in primary cells, enables us to examine *in vivo* the developmental onset of expression of V(D)J recombinase activity and the factors that regulate it. Moreover, once the substrate is recombined VEX continues to be expressed. Because VEX acts a permanent marker of cells that have, or had, recombinase activity, we also have the opportunity to evaluate the contribution of recombinase⁺ lymphoid progenitors to the B, T, NK, and DC lineages.

Materials and Methods

Mice. C57BL/6 and RAG1^{-/-} (C57BL/6 background) mice were obtained from the Jackson Laboratories. *Erag*^{-/-} mice were provided by Mark Schlissel (University of California, Berkeley, CA) (26). RAG2 GFP NG BAC mice (FVBN) provided by Michel Nussenzweig (The Rockefeller University, New York, NY) (17) were backcrossed to C57BL/6 in our laboratory for 12 generations. All mice were treated humanely in accordance with federal and state government guidelines and UMMS institutional animal committees.

Construction of H2-SVEX Transgenic Mice. The H2-SVEX transgene was constructed by placing the RSS-VEX-RSS fragment (Fig. 1 A) into the H2K (HIL) transgenic vector using a unique NotI restriction site located between the H2 promoter and the H2 exon fragment. The H2K cassette vector expresses genes under the control of the H2K promoter/enhancer and Moloney MuLV enhancer/poly(A), typically at high levels in HSC and all hematolymphoid cells (32–35). Heterologous promoter activation has been shown to be sufficient for directing rearrangement of chromosomal recombination substrates (36). Transgenic mice were made at the UMMS transgenic facility using standard procedures. From the injected C57BL/6 embryos, 13 of 136 mice were positive for the transgene as analyzed by PCR. Of these potential founders, six expressed VEX in peripheral white blood cells and four such mice were used to establish permanent transgenic lines: SB68, SB88, SB110, and SB114. VEX is from MFG-hu-VEX-2 (37–39). The recombination signal sequence (RSS) fragments contain consensus RSS and 16 bp from the murine DFL16.1 coding region (12-RSS) or 17 bp from the murine JH1 coding region (23-RSS) (40).

Cell Sorting and Flow Cytometry. Freshly isolated cells were resuspended to 3×10^7 cells/ml in staining media containing biotin-, flavin-, and phenol red–deficient RPMI 1640 (Irvine Scientific), 10 mM Hepes, pH 7.2, 0.02% sodium azide, 1 mM EDTA, and 3% newborn calf serum and treated with 2.4G2 Fc block for 10 min on ice. Cells were incubated with primary antibodies for 20 min, then washed three times, incubated with streptavidin reagents for 15 min, and then washed three more times. After the final wash, samples were resuspended in 1 μ g/ml propidium iodide to exclude dead cells. Primary antibodies included AA4.1 biotin or APC, B220 APC or Cy5PE or FITC, BP-1 biotin (6C3), CD3 Cy5PE or PE, CD4 Cy5PE or PE, CD8 APC or Cy5PE or PE, CD11b APC (M1/70), CD19 Cy5PE or FITC, CD24 Cascade blue, Alexa 594 or FITC (30-F1), CD43 PE, CD122 biotin or PE, c-kit PE, DX5 biotin or

FITC, Ly6C biotin or FITC, Gr-1 PE (Ly6G; RB6–8C5), IgM biotin or FITC, IL-7R PE (SB/14 or A7R34), Ly49G APC, and NK1.1 biotin or FITC. Secondary reagents were SA-Cy5PE, SA-Alexa 594, SA-Cy7PE or SA-APC. Antibodies were purchased from BD Biosciences, eBioscience, Southern Biotechnology Associates, Inc., or CALTAG and purified and conjugated using established procedures (37). Flow cytometry was performed on a 3 laser, 7 detector DIVA FACS Vantage or a 3 laser 9 detector LSR II (Becton Dickinson). VEX was detected using 407 nm excitation and a 510-nm (10-nm bandpass) filter (37). Data were analyzed with FlowJo software (Tree Star), and all flow cytometric data is presented as 5% contour plots.

DNA Isolation and PCR. Genomic DNA was isolated with the QIAGEN DNeasy kit according to the manufacturer's instructions. PCR amplification was performed with 10 μ l DNA in 25 μ l total volume with 1.6 μ M dNTP (dATP + dTTP + dCTP + dGTP), 2.5 U Taq, 4 mM MgCl₂, 1 \times buffer A (Fisher Scientific), and 4 μ M of each primer. Primers were 2011 (5'), TAAAGTCCACGCAGCCCCGAGA; 2165 (3'), GGTG-GAGAAGGGGCGGAGGGTC; 586 (5'), GCCCGTGCT-GCTGCCCGACA; and 200 (3'), CGCCGTAGCTGAAG-GTGGTC. Primers were used in the following combinations (refer to Fig. 1 A for primer location): H2-SVEX transgene, 2011 and 2165; coding joints (CJs), 200 and 2011; and signal joints (SJs), 586 and 2165. PCR conditions consisted of 30 cycles unless otherwise indicated (94°C for 1 min, 68°C for 30 s, and 72°C for 1 min) in an Eppendorf Mastercycler. PCR products were visualized with ethidium bromine on a 1.5% agarose gel in TBE buffer. Single cell PCR analysis for D-JH rearrangements in NK cells was performed as described previously (23).

Online Supplemental Material. Fig. S1 shows lymphocyte subsets in the four independent H2-SVEX founder lines. Fig. S2 characterizes VEX expression in NK cells from C57BL/6 and RAG1^{-/-} mice. Table S1 reports the sequence of CJs and SJs from independent H2-SVEX rearrangements. Supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20031800/DC1>.

Results

The H2-SVEX Transgenic Recombination Substrate. To detect the developmental onset of V(D)J recombinase activity, we developed a FACS[®] reporter gene system in which V(D)J recombination of a transgenic substrate is indicated by VEX fluorescence. VEX is a variant of GFP (37). The V(D)J recombination substrate H2-SVEX expresses VEX as a consequence of V(D)J recombination. As shown in Fig. 1 A, the VEX gene is initially in the anti-sense orientation and is flanked by V(D)J RSSs. RSSs in this orientation mediate inversion such that after recombination VEX is in the correct orientation for expression. Cells that undergo V(D)J recombination are VEX⁺; these cells are easily detected and quantified by FACS[®].

We examined VEX expression in four independent H2-SVEX transgenic lines, SB68, SB88, SB110, and SB114. All four transgenic lines have the same frequency and number of cells within subpopulations of hematopoietic and lymphoid cells compared with wild-type, indicating that cells that express VEX are not at a developmental or homeostatic disadvantage (Fig. S1, available at <http://www.jem.org/cgi/content/full/jem.20031800/DC1>).

Specificity and Efficiency of the H2-SVEX Recombination Substrate. The H2-SVEX construct is driven by an active H2K (murine MHC class I) promoter/MoMLV enhancer that enables robust transgene expression and, presumably, accessibility in all hematopoietic cells (32, 33, 35). Characterization of VEX expression in primary lymphoid tissue demonstrates the specificity and efficiency of the H2-SVEX recombination substrate. Within the B lymphocyte lineage, 94% of immature B220⁺IgM⁺CD24^{hi}Ly6C⁻DX5⁻ bone marrow B cells (23) and 84–86% of CD19⁺IgM⁺ splenic B cells (Fig. 1 B) are VEX⁺, indicating efficient recombination of the substrate in both the SB110 and SB88 H2-SVEX lines. VEX expression is also high among splenic T lymphocytes since 91–93% of CD3⁺ T cells are VEX⁺. By contrast, 0.5% of splenic myeloid cells and 0.3–0.5% of granulocytes are VEX⁺, suggesting that H2-SVEX is not rearranged in nonlymphoid lineages. Importantly, Fig. 1 B shows that the percentage of VEX⁺ B and T cells is nearly identical between the two independent founder lines depicted (SB88 and SB110), indicating position-independent regulation of recombination.

To demonstrate directly that H2-SVEX undergoes V(D)J recombination, we bred H2-SVEX mice to RAG1^{-/-} mice. H2-SVEX recombination in both SB110 and SB88 transgenic B220⁺ bone marrow cells is abolished in the absence of RAG1 as the frequency of detectable VEX⁺ cells is diminished from 66% in wild-type H2-SVEX to 1.3% in H2-SVEX RAG1^{-/-} animals, comparable to background fluorescence detected in C57BL/6 and RAG1^{-/-} nontransgenic controls (Fig. 1 C and not depicted).

Together, these data demonstrate that H2-SVEX rearrangement (1) is restricted to the lymphoid lineages, (2) re-

combines efficiently as a V(D)J recombination substrate, with frequencies of rearrangement approaching 90% within splenic lymphocytes, and (3) completely depends on RAG1 expression.

H2-SVEX Recombination Has Expected Molecular Characteristics of V(D)J Recombination and Is Accurately Reflected by VEX Expression. We examined by direct molecular analysis whether CJs and SJs, characteristic V(D)J recombination products (Fig. 1 A), are detectable after H2-SVEX recombination. The gels in Fig. 2 A depict PCR amplification of transgene-specific CJ and SJ recombination products (first and second rows), the transgene itself independent of recombination (third row) and, as a loading and PCR control, endogenous H2K (fourth row) in H2-SVEX transgenic or C57BL/6 nontransgenic controls. The H2-SVEX-specific CJs and SJs are detectable in splenocytes from two H2-SVEX founder lines, SB88 (Fig. 2 A, lane 2) and SB110 (lane 7), but not C57BL/6 (lane 1). Sequencing of recombined H2-SVEX products from primary lymphocytes confirms that the transgene undergoes multiple, independent rearrangements that produce highly variable CJs (all 12 joints cloned were unique) and relatively conserved SJs (8 out of 12 joints cloned were perfect; Table S1, available at <http://www.jem.org/cgi/content/full/jem.20031800/DC1>). Neither CJs nor SJs are detectable in SB88 brain (Fig. 2 A, lane 4), and CJ products are only very weakly detectable in SB110 kidney (lane 6) relative to C57BL/6 (lanes 3 and 5). The latter is likely due to low levels of lymphocyte contamination within this highly vascularized organ, since we can readily amplify rearranged TCR genes from kidney (not depicted). Moreover, CJ formation is readily detectable in sorted VEX⁺ but not VEX⁻

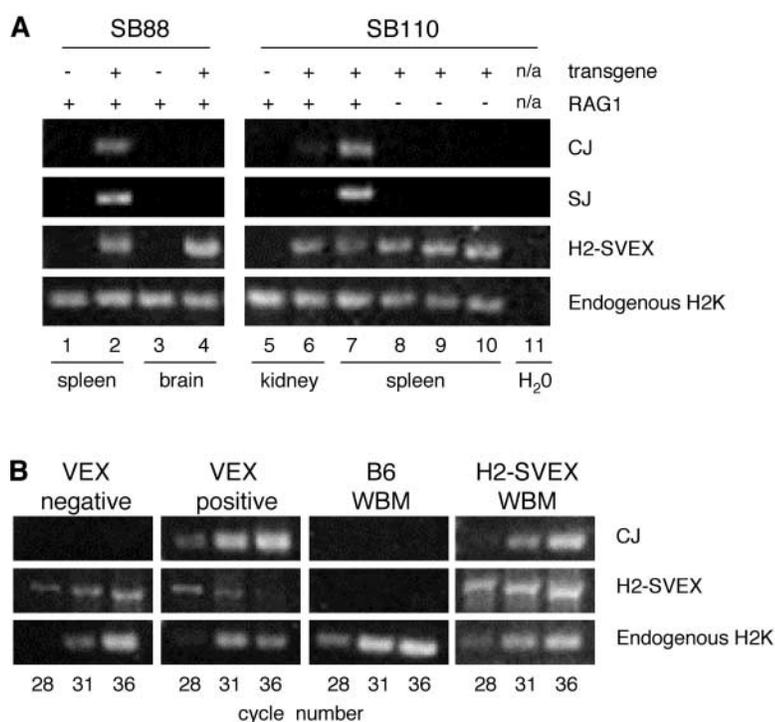


Figure 2. (A) PCR analysis of H2-SVEX V(D)J recombination. H2-SVEX recombination products were examined in two different transgenic lines, SB88 (left panel) or SB110 (right panel), which express or lack RAG as indicated. Nontransgenic C57BL/6 animals are presented as a control. Primers specific to the transgene were used to detect the recombination products CJs and SJs in the indicated tissues. Primers that bind within the murine H2K gene were used to amplify the 1-kb SVEX cassette in transgenic mice (independent of recombination; labeled H2-SVEX in the figure) or to amplify a distinct 250-bp PCR product in both transgenic and wild-type mice (labeled endogenous H2K) derived from the endogenous H2K gene. Amplification of endogenous H2K serves as a positive control confirming the presence of template DNA. (B) Semiquantitative PCR analysis of H2-SVEX recombination. SB88 H2-SVEX bone marrow was FACS[®] sorted into VEX⁻ and VEX⁺ populations. DNA from an equivalent number of cells was subject to PCR analysis for H2-SVEX transgene-specific CJs, the H2-SVEX transgene, and endogenous H2K. The PCR conditions were designed to give linear amplification for CJs, and cell cycle number is indicated. The data are representative of two to five experiments using cells from independent sorts.

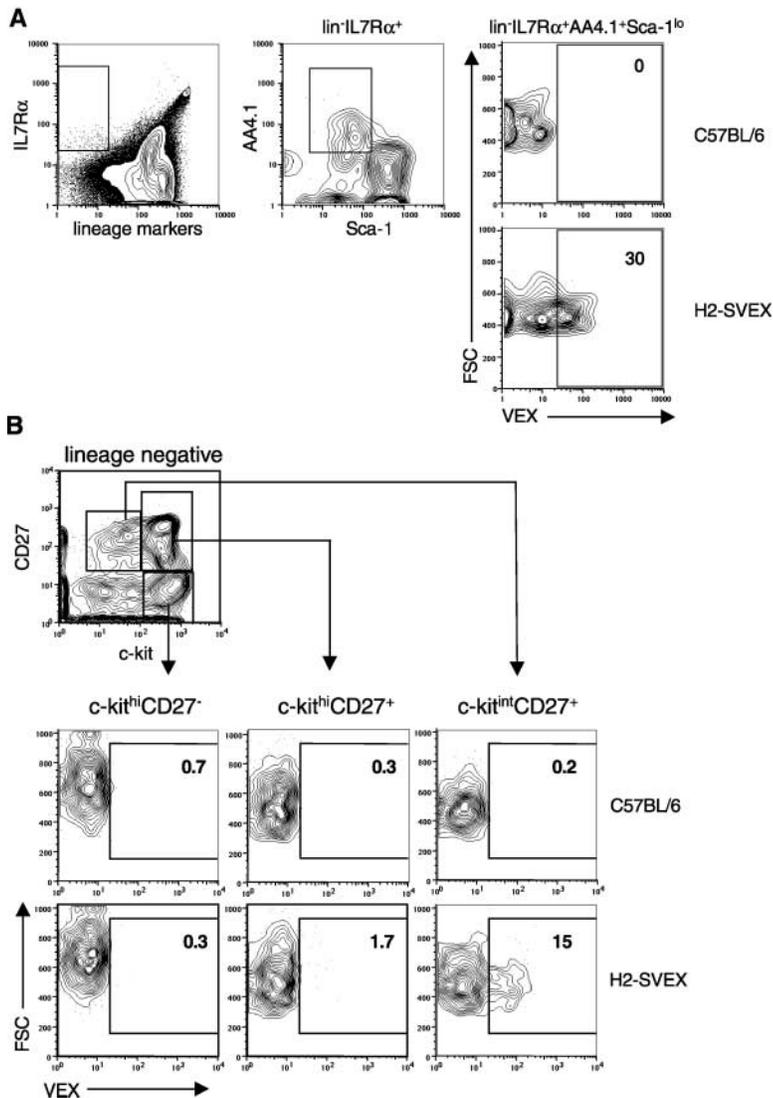


Figure 3. V(D)J recombinase activity in multipotent hematopoietic progenitors. (A) V(D)J recombinase activity in CLPs. The lin⁻ subset (B220, CD11b, GR-1, Ter119, CD3) of bone marrow obtained from SB88 H2-SVEX transgenic or C57BL/6 control mice was examined for VEX expression in IL-7R α ⁺AA4.1⁺Sca-1^{lo/-} cells. (B) The lin⁻ subset of bone marrow obtained from SB110 H2-SVEX transgenic or C57BL/6 control mice was examined for VEX expression as a function of c-kit and CD27. The data are representative of two to five independent experiments.

BM cells from SB88 H2-SVEX transgenics even after 36 cycles of amplification (Fig. 2 B). These observations indicate that FACS[®] detection of VEX accurately reflects V(D)J recombination of the H2-SVEX transgene and provides strong support for the sensitivity of this GFP-based reporter construct to reflect *in vivo* recombination. By contrast, neither CJs nor SJs are detectable in any of three independent H2-SVEX RAG1^{-/-} mice (Fig. 2 A, lanes 8–10), confirming that H2-SVEX recombination depends on RAG activity and, hence, the V(D)J recombinase.

Expression of V(D)J Recombinase Activity in Early Hematopoietic Cells. We have demonstrated that V(D)J recombinase activity is detectable in the earliest B and T lineage progenitors before IgH (and TCR) recombination (23). One of the major implications of this result is that the recombinase may be expressed even before B lineage commitment. To explore this possibility, we examined VEX expression within the lin⁻IL-7R α ⁺AA4.1⁺Sca-1^{lo} subset of bone marrow, which contains hematopoietic precursors that retain lymphoid (B, T, NK, and DC), but not myeloid

developmental potential (27, 28), and are designated as common lymphoid progenitors or CLPs. VEX expression is readily detectable in 30–45% of CLPs (Fig. 3 A and not depicted; see also Fig. 6).

We then examined recombinase activity in earlier hematopoietic progenitor subsets that retain the capacity for self-renewal. Recombinase activity is not detectable in lin⁻ckit^{hi}CD27⁻ progenitors (Fig. 3 B, left). However, VEX expression is detectable in a minor subset (1.7%) of ckit^{hi}CD27⁺ progenitors, cells that include the lymphoid-restricted early lymphoid progenitor (41), as well as a subset (15%) of ckit^{int}CD27⁺ progenitors (Fig. 3 B, middle and right), cells that include the CLP (27). Together these data suggest that the V(D)J recombinase is first active at low levels in hematopoietic progenitors after loss of myeloid developmental potential, and recombinase activity increases throughout the earliest stages of B lymphocyte development (23).

V(D)J Recombinase Activity in the Natural Killer Cell Lineage. In addition to B and T lymphocytes, the CLP gives rise to NK and DC, and it is possible that some VEX⁺

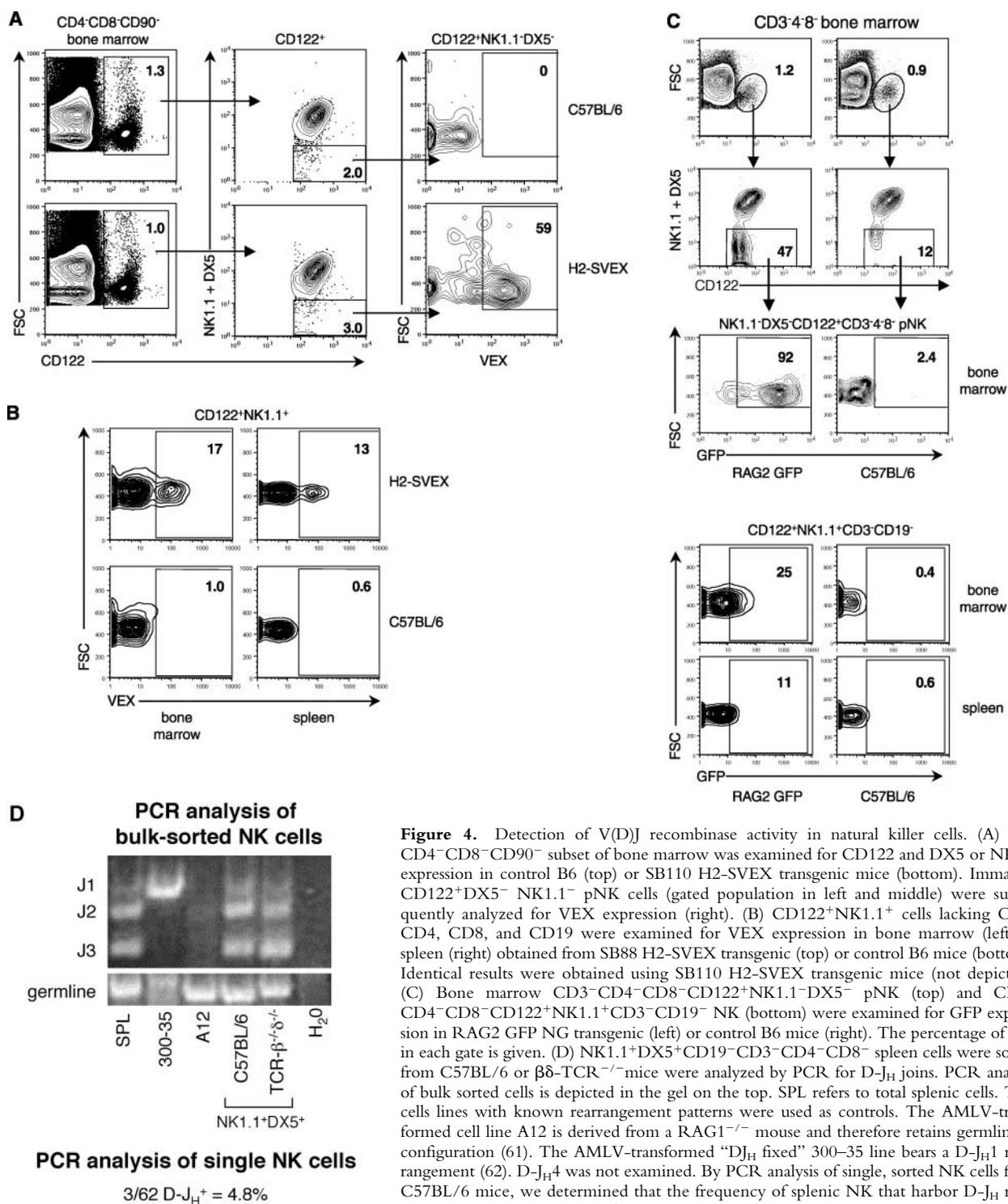


Figure 4. Detection of V(D)J recombinase activity in natural killer cells. (A) The CD4⁻CD8⁻CD90⁻ subset of bone marrow was examined for CD122 and DX5 or NK1.1 expression in control B6 (top) or SB110 H2-SVEX transgenic mice (bottom). Immature CD122⁺DX5⁻ NK1.1⁻ pNK cells (gated population in left and middle) were subsequently analyzed for VEX expression (right). (B) CD122⁺NK1.1⁺ cells lacking CD3, CD4, CD8, and CD19 were examined for VEX expression in bone marrow (left) or spleen (right) obtained from SB88 H2-SVEX transgenic (top) or control B6 mice (bottom). Identical results were obtained using SB110 H2-SVEX transgenic mice (not depicted). (C) Bone marrow CD3⁻CD4⁻CD8⁻CD122⁺NK1.1⁻DX5⁻ pNK (top) and CD3⁻CD4⁻CD8⁻CD122⁺NK1.1⁺CD3⁻CD19⁻ NK (bottom) were examined for GFP expression in RAG2 GFP NG transgenic (left) or control B6 mice (right). The percentage of cells in each gate is given. (D) NK1.1⁺DX5⁺CD19⁻CD3⁻CD4⁻CD8⁻ spleen cells were sorted from C57BL/6 or $\beta\delta$ -TCR^{-/-} mice and analyzed by PCR for D-J_H joins. PCR analysis of bulk sorted cells is depicted in the gel on the top. SPL refers to total splenic cells. Two cell lines with known rearrangement patterns were used as controls. The AMLV-transformed cell line A12 is derived from a RAG1^{-/-} mouse and therefore retains germline Ig configuration (61). The AMLV-transformed “DJ_H fixed” 300–35 line bears a D-J_H1 rearrangement (62). D-J_H4 was not examined. By PCR analysis of single, sorted NK cells from C57BL/6 mice, we determined that the frequency of splenic NK that harbor D-J_H rearrangements was 4.8% (3 D-J_H⁺ cells out of 62 NK cells analyzed) as indicated on the bottom. The data in A–C are representative of two to five independent experiments. The data in D are representative of at least two experiments using independently sorted cells.

CLPs may give rise to these two lineages. Because VEX permanently marks cells that have expressed the V(D)J recombinase, we had the opportunity to examine whether some NK and DC have a history of recombinase activity. We first examined VEX in NK precursors (pNK; CD122⁺

CD4⁻CD8⁻CD90⁻DX5⁻) (42, 43). Approximately 60% of pNK from H2-SVEX mice express VEX versus 0% from control B6 (Fig. 4 A, right). We also examined expression of *rag2* transcription in this subset using the NG RAG2 transgenic reporter line in which GFP replaces the major

exon of RAG2 (17). The pNK population from these reporter mice had high levels of GFP expression (Fig. 4 C, top, third row, left panel), indicative of RAG2 transcription, supporting the idea that the pNK may have (or may have had) an active V(D)J recombinase.

Examination of more mature NK cells confirms that NK cells display evidence of recombinase activity. Within the CD3⁻CD8⁻CD19⁻ subset, VEX expression in CD122⁺NK1.1⁺ NK cells is readily detectable in both bone marrow and spleen (Fig. 4 B). Decreased expression of VEX in mature versus precursor NK cells may reflect down-regulation of reporter transgene expression with progression to this particular lineage or may be due to preferential selection of NK cells that express little or no recombinase activity. VEX is also readily detectable in cells bearing the NK cell-specific Ly49G receptor (Fig. S2, available at <http://www.jem.org/cgi/content/full/jem.20031800/DC1>), and as with the other lymphocytes, recombination of H2-SVEX in NK cells depends on the presence of RAG1 (Fig. S1). We also examined *rag2* expression in mature NK cells and found that GFP is detectable only within a moderate percentage (11–25%) of NK1.1⁺ bone marrow and spleen cells (Fig. 4 C, bottom left) and is quite dull compared with expression in pNK (compare Fig. 4 C, top and bottom). These data suggest that *rag2* is likely to be expressed and active only at a very early stage of NK development rather than during later maturation stages in the bone marrow.

IgH Recombination in NK Cells. To demonstrate that the V(D)J recombinase can recombine antigen receptor loci in NK cells, we took advantage of observations that IgH recombination events can be detected in T cells and thus are not B lineage restricted (44, 45). Specifically, we examined whether D-J_H recombination events could also be found in NK cells. As shown in Fig. 4 D, sorted NK cells from C57BL/6 mice have readily detectable D-J_H joins, suggesting that the V(D)J recombinase is active at endogenous loci in NK cells. Moreover, D-J_H joins are also detectable in NK cells from TCRβδ^{-/-} animals, demonstrating that this result is not due to contamination by either NK T cells or classical T cells, many of which harbor D-J_H joins. To obtain a quantitative estimate of the frequency of NK cells that possess rearrangement events, we examined the frequency of D-J_H joins by single cell PCR analysis. We found that 3/62 (~5%) splenic NK cells have detectable D-J_H joins. Together, these data indicate that the V(D)J recombinase is active at some point in the developmental history of NK cells and can complete the D_H to J_H joining step.

V(D)J Recombinase Activity in the DC Lineage. We extended our observations to examine recombinase expression in the DC lineage, which includes cells that also arise from the CLP. We found that 12% of DC progenitors (28) from H2-SVEX animals express VEX versus 0% of control B6 animals (Fig. 5 A). DC progenitors isolated from RAG2 GFP transgenic mice also express GFP (17). Splenic DCs can be resolved into two populations based on phenotype,

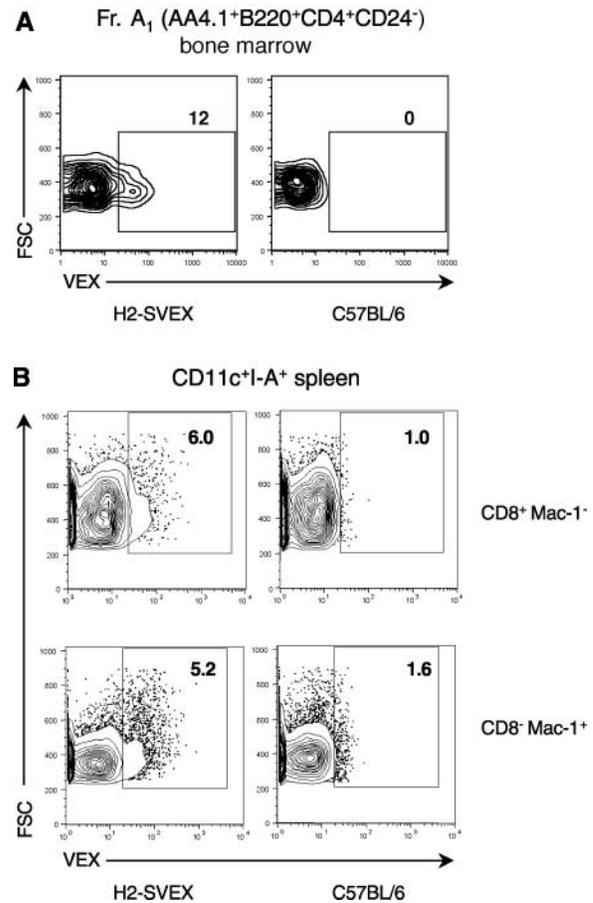


Figure 5. Detection of V(D)J recombinase activity in DCs. (A) VEX expression was examined in AA4.1⁺B220⁺CD4⁺CD24⁻ bone marrow progenitors from SB110 H2-SVEX transgenic (left) or B6 control mice (right). (B) Splenic DCs were enriched by collagenase digestion and Nycodenz gradient centrifugation of tissue obtained from SB110 H2-SVEX transgenic (left) or C57BL/6 control mice (right). The CD11c⁺I-A⁺ subset was examined for expression of CD8, Mac-1, and VEX. The percentage of cells in each gate is given. The data are representative of at least five (A) or two (B) independent experiments, and identical results were obtained with SB88 H2-SVEX transgenics (not depicted).

CD8⁺Mac-1⁻ and CD8⁻Mac-1⁺ (46–48). VEX is detectable in both DC populations as 6% of CD8⁺Mac-1⁻ DCs and 5.2% of CD8⁻Mac-1⁺ DCs from H2-SVEX mice are VEX⁺ versus <2% from B6 controls (Fig. 5 B, bottom). Thus, VEX expression, indicative of V(D)J recombinase activity, is detectable in both NK and DC lineages.

B Lineage-specific Regulation of V(D)J Recombinase Activity in Early Hematopoietic Cells. *rag* expression is controlled by different transacting factors in B versus T cells; yet our data show that the V(D)J recombinase is active in 30–45% of CLPs, cells which retain the potential to produce both B and T cells. Therefore, we wondered whether lineage-specific regulation of recombination would be evident in CLPs. The *Erag* enhancer element, located ~22 kb upstream of the *rag2* gene, profoundly influences expression of both *rag1* and *rag2* in a B lineage-specific manner (26). *Erag*^{-/-} animals have a twofold reduction in *rag2* transcripts

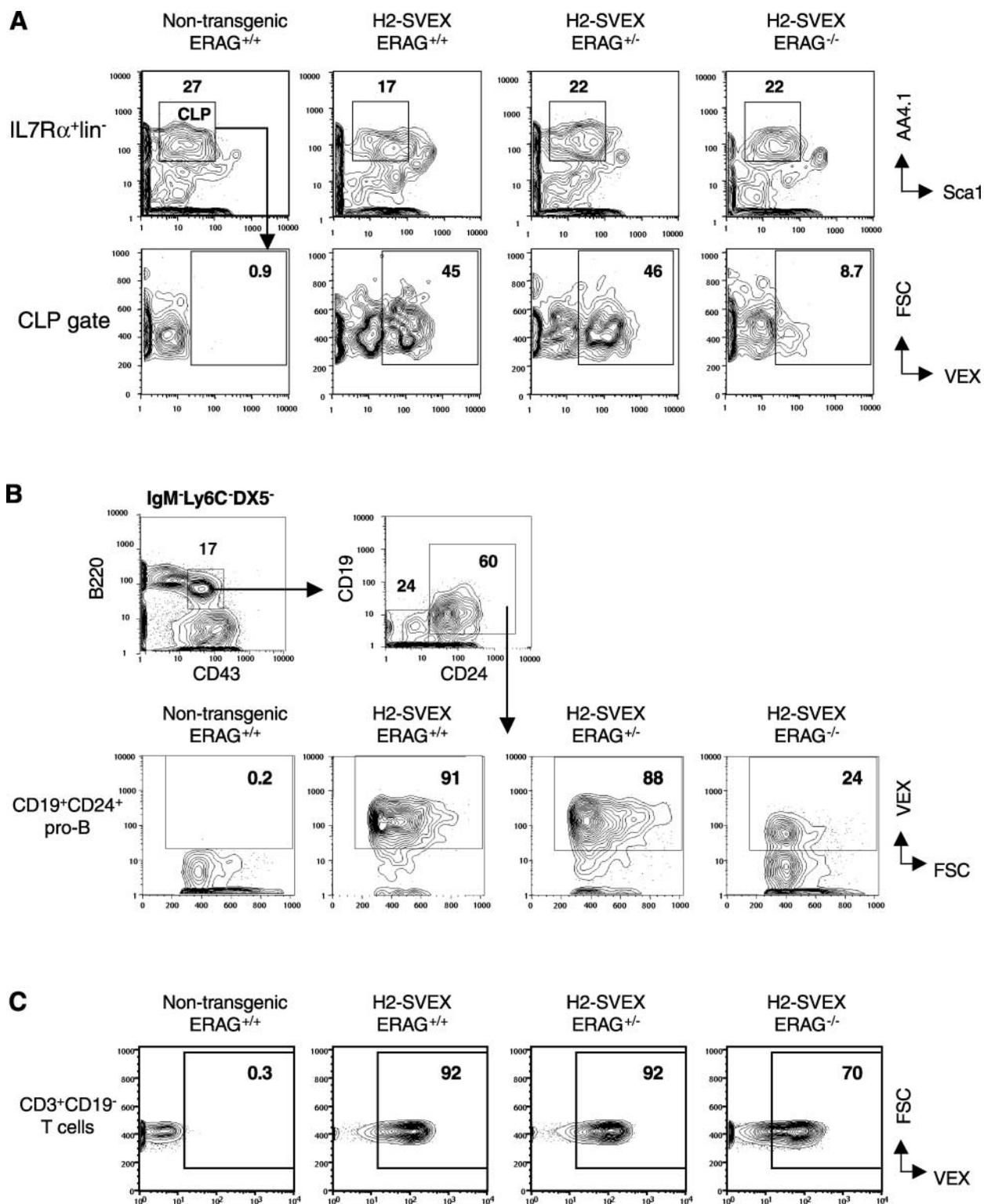


Figure 6. Regulation of V(D)J recombinase activity in multipotential lineage progenitors. (A) Bone marrow from SB110 H2-SVEX animals that were *Erag*^{+/+}, *Erag*^{+/-}, or *Erag*^{-/-} was stained for IL-7R α ⁺AA4.1⁺Sca-1^{lo}/lin⁻ CLPs (top), and cells within the CLP gate were examined for VEX expression (bottom). Identical results were obtained when wild-type SB110 and SB88 H2-SVEX mice were compared (not depicted). (B) VEX expression was analyzed in B220⁺CD43⁺DX5⁻Ly6C⁻IgM⁻ CD19⁺CD24⁺ pro-B cells (bottom row). (C) VEX expression was analyzed in CD3⁺CD19⁻ splenic T cells. The percentage of each population within the gate is given. The data are representative of two to five independent experiments.

and a 10-fold reduction in *rag1* transcripts in pro- and pre-B cells, whereas the T lineage remains unaffected. Therefore, we examined whether *Erag* was important for controlling recombinase activity in CLPs.

Fig. 6 A (top) depicts identification of CLPs ($\text{lin}^{-}\text{IL7R}\alpha^{+}\text{AA4.1}^{+}\text{Sca-1}^{\text{lo}}$) in nontransgenic wild-type animals and in three transgenic mice: H2-SVEX *Erag*^{+/+}, H2-SVEX *Erag*^{+/-}, and H2-SVEX *Erag*^{-/-}. The percentage of AA4.1⁺Sca-1^{lo} CLP cells was similar across all four mice, ranging from 17–26% of $\text{lin}^{-}\text{IL7R}\alpha^{+}$ cells (Fig. 6 A, top). However, whereas 45% of CLPs from H2-SVEX transgenic *Erag*^{+/+} or *Erag*^{+/-} siblings express VEX (Fig. 6 A, bottom), the *Erag*^{-/-} mice had a dramatic (fivefold) reduction in VEX expression (9%; Fig. 6 A, bottom, far right). The decline in recombinase activity in the CLP suggests that B cell-specific regulation of *rag* gene expression already exists even at this early stage of hematopoietic development.

VEX expression is also dramatically reduced in pro-B subsets from H2-SVEX *Erag*^{-/-} transgenics. The percentage of VEX expression in pre-pro-B cells is reduced from 17% in H2-SVEX *Erag*^{+/+} to 6% in *Erag*^{+/-} and to 3.2% in *Erag*^{-/-} animals (not depicted), a fivefold reduction comparable to the magnitude of decrease observed in CLPs. VEX expression in later pro-B cells is reduced from ~90% in H2-SVEX *Erag*^{+/+} and *Erag*^{+/-} mice to 24% in *Erag*^{-/-} (Fig. 6 B). In contrast, VEX expression in CD3⁺ T cells is only modestly reduced (1.2-fold) from 92% H2-SVEX *Erag*^{+/+} and in *Erag*^{+/-} to 70% in *Erag*^{-/-} animals (Fig. 6 C), confirming that V(D)J recombinase activity in the B but not T lineage strongly depends on the *Erag* element.

Discussion

To examine developmental control of V(D)J recombinase activity in single, multipotent hematopoietic progenitors, we developed mice that express the fluorescent V(D)J recombination substrate H2-SVEX. Once recombined, the fluorescent substrate is permanently expressed and provides a useful marker of the developmental history of lymphocytes. We use this marker to show that V(D)J recombinase is active as early as the CLP stage of hematopoietic development, and evidence of this activity is detectable in all four potential progeny lineages: B, T, NK, and DC. By single cell PCR, we also demonstrate the presence of D-J_H rearrangements in 4.8% of splenic NK cells. We take advantage of the sensitivity of H2-SVEX to characterize regulation of V(D)J recombinase expression in CLPs. We demonstrate that recombinase activity in this multipotent population depends on the B lineage enhancer of *rag* expression, *Erag*. This surprising result demonstrates that lineage-specific regulation of *rag* is already established in this multipotent progenitor and suggests one mechanism underlying recent observations that the CLP may be biased toward a B rather than T lineage fate.

V(D)J Recombinase Activity in the CLP. Detection of V(D)J recombinase activity as early as the CLP stage of hematopoietic development extends previous findings that

RAG is expressed not only in progenitors that retain the capacity to produce B lymphocytes and NK cells (41) but also in fraction A₁ DC precursors (17). These results were unexpected because recombinase expression was previously considered restricted to committed B and T cell progenitors. Initiation of V(D)J recombinase expression in CLPs before progression to the B or T lymphocyte lineage is consistent with evidence that hematopoietic development can be very plastic. For example, in *pax5*^{-/-} mice (49) B220⁺ bone marrow cells bearing D-J_H rearrangements and previously considered restricted to the B lineage retain the capacity to give rise to nearly all other blood cells. Even the classically defined CLP ($\text{lin}^{-}\text{IL-7R}^{+}\text{Thy-1}^{-}\text{Sca-1}^{\text{lo}}\text{c-kit}^{\text{lo}}$) that produces B, T, and NK cells has latent myeloid potential that is induced by ectopic expression of IL-2 and GM-CSF receptors (50).

V(D)J Recombinase Activity in NK Cells. Our analysis of NK and DC cells indicates that V(D)J recombinase activity in the lymphoid compartment is not restricted to the B or T lineage (Figs. 4 and 5). The intriguing possibility that the V(D)J recombinase operates in cells other than B and T progenitors finds additional support in observations that (1) *rag1* transcription is detectable in *c-kit*^{hi}*Sca-1*^{hi}*CD27*⁺ progenitors that give rise to both lymphocytes and NK cells (41), (2) incomplete TCR- β rearrangements are detectable at low levels in some murine NK cells (51), and (3) D-J_H rearrangements are detectable in a subset of murine plasmacytoid and thymic DCs (52). Whether the V(D)J recombinase recombines loci other than IgH (Fig. 4 D) and TCR in the NK lineage remains unknown.

Expression of recombinase activity and, hence, D-J_H rearrangements in NK may simply be a developmental relic stemming from recombinase activity in the CLP. The recombinase may be expressed in CLPs that have not yet committed to a specific lymphoid lineage fate (that is, B, T, or NK, or DC) and may be repressed in more committed NK cell or DC progenitors. High expression of the RAG2 GFP transgenic reporter in pNKs but not in later stages of NK development (Fig. 4 C) indicates that this repression occurs after the pNK stage. Moreover, our observation that pNK derived from *Erag*^{-/-} mice have normal levels of VEX expression (not depicted) even though recombinase activity in the CLP is significantly decreased (Fig. 6), suggests that V(D)J recombination could take place during the earliest stages of NK cell development. Numerous malignancies involving both NK leukemias and lymphomas have been identified (53–55). Because V(D)J recombination is associated with chromosomal transposition *in vitro* (56) and *in vivo* (57) as well as chromosomal translocations (58), our observations raise the possibility that recombinase expression in pNK cells may contribute to some of these forms of oncogenesis.

Regulation of V(D)J Recombinase Activity in Multipotent Hematopoietic Progenitors. Given our observation that a proportion of CLPs from H2-SVEX mice express the recombinase, we examined whether lineage-specific regulation of recombination would be evident at this early stage of hematopoietic development. The *Erag* enhancer controls *rag*

expression in B but not T precursors (26). Surprisingly, we found that *Erag* also controls recombinase activity in CLPs as deletion of this enhancer reduced VEX from 45% in wild-type mice to 8.7% in the *Erag*^{-/-} mice (Fig. 6). Thus, the B lineage-specific control exerted by the *Erag* enhancer is apparent even in multipotential progenitors. These observations predict that the CLP, or the upstream (as yet uncharacterized) pre-CLP (59), is the target of inductive signals required for *rag* expression (60).

The observation that the *Erag* enhancer influences recombinase activity in CLPs has implications for our understanding of hematopoietic cell development. Initial models predicted that CLPs from unmanipulated mice have an equal chance of adopting a B cell or a T cell fate in vivo (27, 28). However, a recent report shows that *ikaros* knockout mice lacking CLPs are nonetheless able to generate near normal frequencies of early thymic T lineage progenitors (29), suggesting that CLPs are early B lineage progenitors that do not contribute significantly to thymic T cell development despite a residual T lineage potential. Our data are consistent with this hypothesis and provide the first molecular evidence supporting the idea that the BM CLP may have a default B cell fate in vivo.

In conclusion, the broad utility of H2-SVEX recombination substrate lies in the fact that it (1) sensitively quantifies recombinase activity in vivo, (2) enables analysis at the single cell level, supporting characterization even of very rare progenitor populations, and (3) can be assayed simultaneously with other GFP transgenic reporters. As one example, we successfully quantified recombinase activity in CLPs (Fig. 3 A), a progenitor population representing <0.1% of total bone marrow cells (28), and characterized the role of the *Erag* enhancer in this population. Our approach is broadly generalizable since H2-SVEX mice can be readily used in combination with other genetically mutated strains that affect B and T lymphocyte development.

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