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

Lisa Borghesi, Lih-Yun Hsu, Juli P. Miller, Michael Anderson, Leonard Herzenberg, Leonore Herzenberg, Mark S. Schlissel, David Allman, and Rachel M. Gerstein

1Molecular Genetics and Microbiology, University of Massachusetts Medical School (UMMS), Worcester, MA 01655
2Department of Molecular and Cell Biology, University of California, Berkeley, Berkeley, CA 94720
3Department of Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, Philadelphia, PA 19104
4Department of Genetics, Stanford University School of Medicine, Stanford, CA 94305

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 relocalized 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).}

The online version of this article contains supplemental material.

† Dr. Michael Anderson died on September 20, 2002.

Address correspondence to Rachel M. Gerstein, Molecular Genetics and Microbiology, University of Massachusetts Medical School, 55 Lake Ave. North, S5-714, Worcester, MA 01655. Phone: (508) 856-1044; Fax: (508) 856 5920; email: rachel.gerstein@umassmed.edu

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 \textit{rag2} is sufficient for both complementing lymphocyte development in \textit{rag2}^{-/-} animals (24) and controlling normal expression of \textit{rag1} and \textit{rag2} in B and T lineage progenitors (17). At least two enhancers have been identified within this locus, one at \textasciitilde8 kb and a second at \textasciitilde22 kb upstream of \textit{rag2} (25, 26). The latter of these, identified as \textit{E\textsubscript{rag}}, is particularly interesting because it exhibits B lineage specificity. Mice lacking \textit{E\textsubscript{rag}} have severely reduced expression of \textit{rag1} and \textit{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 \textit{E\textsubscript{rag}} 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 multipotent progenitors may not contribute to each lineage equally. Specifically, \textit{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 \textit{lin}^{-}\textit{ckit}^{-} progenitors can produce either CD19\textsuperscript{+} B cells or DX5\textsuperscript{+} NK cells (30). B lineage precursors share developmental markers with DC precursors (28), and CD19\textsuperscript{+} B lymphocytes cultured in vitro can give rise to DEC205\textsuperscript{+}CD11c\textsuperscript{+} 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 multipotent hematopoietic progenitors and their development, we used a transgenic H2-SVEX substrate containing 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). (A) Splenocytes from SB110 and SB88 H2-SVEX animals were stained with antibodies to detect CD19\textsuperscript{+}IgM\textsuperscript{+} B cells, CD3\textsuperscript{+} T cells, CD11b\textsuperscript{+}CD3\textsuperscript{-}CD19\textsuperscript{-}NK1.1\textsuperscript{-} myeloid cells, or Gr-1\textsuperscript{-}CD19\textsuperscript{-}CD3\textsuperscript{-}NK1.1\textsuperscript{-} granulocytes and subsequently examined for VEX expression. The percentage of VEX\textsuperscript{+} cells in the gate is given, and outliers are shown. (C) H2-SVEX recombination depends on RAG1. B220\textsuperscript{+} B cells in the bone marrow were examined for VEX expression in H2-SVEX SB110, H2-SVEX RAG1\textsuperscript{-/-}, RAG\textsuperscript{--}, and non-transgenic C57BL/6 control mice. The percentage of VEX\textsuperscript{+} cells in the gate is given. H2-SVEX RAG1\textsuperscript{-/-} 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\textsuperscript{-/-} 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. Erg−/− 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 UMSMS 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 re-arrangement of chromosomal recombination substrates (36). Transgenic mice were made at the UMSMS 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 transgenic lines: SB68, SB88, SB110, and SB114. VEX is from MFG-hu-VEX-2 (37–39). The permanent transgenic lines: SB68, SB88, SB110, and SB114.

Results

The H2-SVEX Transgenic Recombination Substrate. To detect the developmental onset of V(D)J recombination 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 continues to be expressed. Because VEX acts as 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.

FITC, Ly5C 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 LSRII (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 μl DNA in 25 μl total volume with 1.6 μM dNTP (dTTP + dATP + dCTP + dGTP), 2.5 U Taq, 4 mM MgCl2, 1× buffer A (Fisher Scientific), and 4 μM of each primer. Primers were were 2011 (5′), TAAAGTCCACGCAAGCAGA; 2165 (3′), GTGGTGAGAAAGGGCGGAGGGTC; 586 (5′), GCCCGGTGCT-CGTCGCCGACA; and 200 (3′). CGCGTGACTGAAAG-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 2165; 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 bromide 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.
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+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 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− cells from independent sorts.

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 with 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.
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-7Rα+AA4.1+Sca-1h 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+CD27− progenitors (Fig. 3 B, left). However, VEX expression is detectable in a minor subset (1.7%) of ckit+CD27+ progenitors, cells that include the lymphoid-restricted early lymphoid progenitor (41), as well as a subset (15%) of ckit+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).

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-7Rα+AA4.1+Sca-1h− 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.
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⁻ NK1.1⁻) (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-JH 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-JH joins, suggesting that the V(D)J recombinase is active in endogenous loci in NK cells. Moreover, D-JH joins are also detectable in NK cells from TCRβ−/− animals, demonstrating that this result is not due to contamination by either NK T cells or classical T cells, many of which harbor D-JH joins. To obtain a quantitative estimate of the frequency of NK cells that possess rearrangement events, we examined the frequency of D-JH joins by single cell PCR analysis. We found that 3/62 (≈5%) splenic NK cells have detectable D-JH 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, 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 Erg<sup>++</sup>, Erg<sup>−/−</sup>, or Erg<sup>−/−</sup> was stained for IL-7Rα<sup>+</sup>AA4.1<sup>−</sup>Sca-1<sup>−</sup>lin<sup>−</sup> 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<sup>+</sup>CD43<sup>−</sup>DX5<sup>−</sup>Ly6C<sup>−</sup>IgM<sup>−</sup>CD19<sup>−</sup>CD24<sup>−</sup> pro-B cells (bottom row). (C) VEX expression was analyzed in CD3<sup>+</sup>CD19<sup>+</sup> 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 Enrg was important for controlling recombining activity in CLPs.

Fig. 6 A (top) depicts identification of CLPs (lin-IL7Rα+AA4.1+Sca-1<sup>lo</sup>) in nontransgenic wild-type animals and in three transgenic mice: H2-SVEX Enrg<sup>+/+</sup>, H2-SVEX Enrg<sup>+/−</sup>, and H2-SVEX Enrg<sup>−/−</sup>. The percentage of AA4.1+Sca-1<sup>lo</sup> CLP cells was similar across all four mice, ranging from 17–26% of lin-IL7Rα+ cells (Fig. 6 A, top). However, whereas 45% of CLPs from H2-SVEX transgenic Enrg<sup>+/+</sup> or Enrg<sup>+/−</sup> siblings express VEX (Fig. 6 A, bottom), the Enrg<sup>−/−</sup> mice had a dramatic (fivefold) reduction in VEX expression (9%; Fig. 6 A, bottom, far right). The decline in recombining 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 Enrg<sup>−/−</sup> transgenics. The percentage of VEX expression in pro-B cells is reduced from 17% in H2-SVEX Enrg<sup>+/−</sup> to 6% in Enrg<sup>−/−</sup> and to 3.2% in Enrg<sup>−/−</sup> 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 Enrg<sup>+/−</sup> and Enrg<sup>−/−</sup> mice to 24% in Enrg<sup>−/−</sup> (Fig. 6 B). In contrast, VEX expression in CD3<sup>+</sup> T cells is only modestly reduced (1.2-fold) from 92% H2-SVEX Enrg<sup>+/−</sup> and in Enrg<sup>−/−</sup> to 70% in Enrg<sup>−/−</sup> animals (Fig. 6 C), confirming that V(D)J recombinase activity in the B but not T lineage strongly depends on the Enrg 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 recombinase 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<sub>H</sub> 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 Enrg expression, Enrg. 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<sup>−/−</sup> mice (49) B220<sup>+</sup> bone marrow cells bearing D-J<sub>H</sub> 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 (lin−IL7R<sup>+</sup>Thy-1−Sca-1<sup>lo</sup>kit<sup>lo</sup>) 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<sup>+</sup>Sca-1<sup>lo</sup>CD127<sup>+</sup> 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<sub>H</sub> rearrangements are detectable in a subset of murine plasma cell and thymic DCs (52). Whether the V(D)J recombinase recombines loci other than IgH (Fig. 4 D) and TCR-β 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 Enrg<sup>−/−</sup> 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 recombinase 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 recombinase 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 Enrg enhancer controls rag
expression in B but not T precursors (26). Surprisingly, we found that Erg also controls recombinase activity in CLPs as deletion of this enhancer reduced VEX from 45% in wild-type mice to 8.7% in the Erg<sup>−/−</sup> mice (Fig. 6). Thus, the B lineage–specific control exerted by the Erg enhancer is apparent even in multipotent 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 Erg 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 recombinase 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 Erg enhancer in this population. Our approach is broadly generalizable since H2-SVEX mice can be readily used in combination with other genetically marked animals.

We gratefully acknowledge Ranjan Sen, Michel Nussenzweig, Richard Hardy, Avinash Bhandoola, Janet Staever, Bob Woodland, Madelyn Schmidt, and John Labrie for invaluable assistance with experiments. Naomi Rosenberg (Tufts University School of Medicine, Boston, MA) and Chris Roman (State University of New York–Downstate Medical Center, Brooklyn, NY) kindly provided the 300-35 and A12 cell lines, respectively. B6-TCR<sup>−/−</sup> animals were a kind gift from Ray Welsh (University of Massachusetts Medical School, Worcester, MA) and Eva Szomolanyi-Tsuda (University of Massachusetts Medical School). We thank Dr. Irving Weissman (Stanford University Medical School, Stanford, CA) for the generous gift of the H2K (HII) transgenic vector, the Stanford Shared FACS facility and, in particular, Aaron Kantor for advice and encouragement and Gina Jager for assistance during the early stages of this work. We are also grateful to Joseph Gosselin and Steve Jones for useful advice and assistance with construction of transgenic mice. Finally, we thank the UMass Flow Cytometry Facility, UMass Nuclear Acid Facility, and UMass Transgenic Facility for their excellent help.

This work is supported by National Institutes of Health grant RO1 AI043534-03 (to R.M. Gerstein), National Institutes of Health grant RO1 HL48702 (to M.S. Schlisel), training grant AI007349-14 (L. Borghesi), and NIDDK 5 P30 DK32520 to the UMMS Diabetes and Endocrinology Center.

Submitted: 16 October 2003
Accepted: 19 December 2003

References


30. Kouro, T., V. Kumar, and P.W. Kincade. 2002. Relation-