E47 is required for V(D)J recombinase activity in common lymphoid progenitors

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Common lymphoid progenitors (CLPs) are the first bone marrow precursors in which V(D)J recombinase activity is up-regulated. Here, we show that loss of the transcription factor E47 produces a reduced CLP population that lacks V(D)J recombinase activity and D–JH rearrangements in vivo. Apart from a profound arrest before the pro–B cell stage, other downstream lymphoid progeny of CLPs are still intact in these mice albeit at reduced numbers. In contrast to the inhibition of recombinase activity in early B lineage precursors in E47-deficient animals, loss of either E47 or its cis-acting target Erag (enhancer of rag transcription) has little effect on recombinase activity in thymic T lineage progenitors. Taken together, this work defines a role for E47 in regulating lineage progression at the CLP stage in vivo and describes the first transcription factor required for lineage-specific recombinase activity.

Despite the essential role of the V(D)J recombinase in catalyzing antigen receptor loci rearrangement, the molecular mechanisms that regulate recombinase activity in hematopoietic progenitors remain unknown. The rag1/2 components of the V(D)J recombinase initiate recombination by cleaving the DNA at Ig or TCR loci. rag expression is first detectable in a rare subset of lin− sca− kithi (LSK) progenitors (1). Both rag transcription and V(D)J recombinase activity are then up-regulated in bone marrow common lymphoid progenitors (CLPs), cells that efficiently produce B lymphocytes, and in early thymic progenitors (ETPs), cells that efficiently produce T lymphocytes (2, 3).

Conflicting studies highlight a potentially important role for the E2A gene products E47 and E12 as regulators of V(D)J recombinase activity during the earliest stages of lymphocyte development. First, whole bone marrow from E2A- or E47-null mice lacks detectable rag transcripts and D-JH rearrangements, suggesting a requirement for E2A activity in IgH recombination during B cell development (4, 5). However, both E2A- and E47-null mice also have a complete block in early B cell production. Thus, it is unclear whether the lack of rag expression and D-JH rearrangements is due to a specific block in recombination processes or to the absence of B lineage progenitors. Second, retroviral reconstitution of E47 in long-term cultured E2A-deficient hematopoietic progenitors restores both rag1 and rag2 expression in vitro (6), and ectopic expression of E12 in the 70Z/3 cell line promotes expression of rag1 (7). However, in other cell lines, expression of E2A was not sufficient to initiate V(D)J recombination in the absence of rag cotransfection, rendering it unclear whether E2A is a major regulator of rag transcription (8–10). Third, E2A binds to Erag, an enhancer of rag expression whose activity is required for V(D)J recombinase activity at the CLP stage of development (2, 11). E2A is expressed at low levels in LSKs and is highly up-regulated during progression to the B lineage (1, 12), but it remains unknown whether E2A activity is required for recombination in CLPs.

E2A initiates a key transcriptional cascade involving early B cell factor (EBF) and pax5 that leads to the expression of lineage-specific genes required for B cell development and survival, including CD19, IgL, mb-1, B29, A5, VpreB, and IL7R (13–15). Long-term culture with IL-7 restores detectable D-JH joints in hematopoietic precursors from E2A-null mice, indicating that these cells can undergo V(D)J recombination under specific culture conditions (6). There are two possible explanations for this observation. First, supportive culture
conditions may enable survival of D-JH\(^{+}\) progenitors that would have died in vivo due to a developmental block. Second, supportive culture conditions may permit recombination events that are unable to occur in vivo due to the absence of requisite V(D)J recombination machinery. It would be useful to identify which hematopoietic progenitor subsets are still intact in mice lacking E2A gene products to determine the role of this transcription factor in regulating recombination initiation in vivo. To directly address this issue, we characterized the presence of specific hematopoietic progenitor populations in E47-deficient mice and analyzed recombination activity within these precursor subsets using an in vivo fluorescent reporter of V(D)J recombinase activity.

**RESULTS**

**E47-null mice completely lack pro–B cells**

Because the precise stage at which B lineage development is blocked in E47- and E2A-null mice remains undefined, it is unclear whether the lack of detectable V(D)J rearrangements in fetal liver and bone marrow (4, 16) is due to a block in recombination or to the absence of the relevant developmental subsets in which V(D)J recombination occurs. Therefore, we characterized the presence of the earliest B lineage progenitors in mice lacking E47. AA4.1\(^{+}\) B220\(^{+}\) B lineage progenitors in the bone marrow can be resolved into pre-pro–B (CD19\(^{-}\)CD24\(^{hi}\)) and pro-/pre-B (CD19\(^{+}\)CD24\(^{lo}\)) subsets (Fig. 1 A, top and middle rows). For this analysis, we excluded DX5\(^{+}\) NK cells, CD4\(^{+}\) DC progenitors, and Ly6C\(^{+}\) myeloid and plasma cells (2, 17, 18), populations that contribute significant contamination to the B lineage progenitor gates.

We found that pro-/pre-B cells were virtually undetectable in E47-null mice. This population was reduced from 65–82% in E47-heterozygous animals to 0–0.2% in E47-deficient animals (Fig. 1 A, middle row, and not depicted). In contrast to the complete absence of pro–B cells, pre-pro–B cells are detectable in E47-null mice. However, pre-pro–B cells are reduced three- to fourfold in absolute cell number from 19,655 ± 19,164 (\(n = 10\)) in E47-heterozygous mice to 5,086 ± 5,743 (\(n = 10\)) in E47-null mice (Fig. 2). The cytokine receptors flt3 and c-Kit, which are expressed on the surface of the earliest B lineage precursors and down-regulated thereafter (19, 20), were detectable at comparable levels on pre-pro–B cells from E47-null versus E47-heterozygous animals (Fig. 1 B and not depicted).

Because E47 might regulate expression of the AA4.1 epitope, we confirmed the developmental block between pre-pro– and pro–B cells using an analysis scheme that does not rely on discrimination of the AA4.1 antigen. Within the B220\(^{+}\)CD43\(^{+}\)CD4\(^{-}\)DX5\(^{-}\)Ly6C\(^{-}\) IgM\(^{-}\) subset, we found that CD19\(^{-}\)CD24\(^{lo}\) pre–pro–B cells, but not CD19\(^{+}\)CD24\(^{hi}\) pro–B cells, were detectable in E47-null mice (Fig. 1 C, middle row). Thus, using two different schemes, we found that E47-deficient animals completely lack the pro– and pre–B populations.

We examined recombinase activity in pre–pro–B cells from E47-null mice by crossing these animals to the H2-SVEX recombination substrate reporter line. In these mice, VEX, a spectral variant of GFP, is a sensitive in vivo reporter of V(D)J recombinase activity (2, 21). We found that VEX expression was reduced 65–85% in E47-null pre–pro–B cells defined according to either phenotypic model in which CD19\(^{-}\)CD24\(^{hi}\) pre–pro–B cells and CD19\(^{+}\)CD24\(^{lo}\) pro–/pre–B cells within the B220\(^{+}\)AA4.1\(^{+}\) subset. Contaminating populations expressing DX5, Ly6C, IgM, or CD4 were excluded from the analysis. Pre–pro–B cells were then analyzed for VEX expression. (B) Pre–pro–B cells were examined for flt3 expression. (C) B lineage progenitors were identified according to an alternative phenotypic model in which CD19\(^{-}\)CD24\(^{hi}\) pre–pro–B cells and CD19\(^{+}\)CD24\(^{lo}\) pro–B cells are identified within the B220\(^{+}\)CD43\(^{-}\) subset. As in A, cells bearing DX5, Ly6C, IgM, or CD4 are excluded from the analysis. B cell progenitor populations were subsequently analyzed for VEX expression. The data are representative of three to five independent experiments.

Figure 1. The B lineage defect in E47-null mice. (A) Bone marrow from E47-heterozygous or E47-null mice crossed to the H2-SVEX (SB110) background was stained with antibodies to detect CD19\(^{-}\)CD24\(^{hi}\) pre–pro–B cells and CD19\(^{+}\)CD24\(^{lo}\) pro–/pre–B cells within the B220\(^{+}\)AA4.1\(^{+}\) subset. Contaminating populations expressing DX5, Ly6C, IgM, or CD4 were excluded from the analysis. Pre–pro–B cells were then analyzed for VEX expression. (B) Pre–pro–B cells were examined for flt3 expression. (C) B lineage progenitors were identified according to an alternative phenotypic model in which CD19\(^{-}\)CD24\(^{hi}\) pre–pro–B cells and CD19\(^{+}\)CD24\(^{lo}\) pro–B cells are identified within the B220\(^{+}\)CD43\(^{-}\) subset. As in A, cells bearing DX5, Ly6C, IgM, or CD4 are excluded from the analysis. B cell progenitor populations were subsequently analyzed for VEX expression. The data are representative of three to five independent experiments.
vivo: (a) regulating pre–pro– and pro–B cell development and (b) controlling V(D)J recombination.

E47 and Erag are not required for recombinase activity in T lineage progenitors

We have previously shown that recombinase activity in B but not T cells depends on the Erag enhancer of \( \text{rag} \) expression, which is a target of E2A (2, 11). In these experiments, we had examined the effects of Erag deletion on VEX expression in splenic T cells, taking advantage of the fact that VEX is a permanent marker of V(D)J recombination that occurs in the thymus (2). To directly assess the contribution of E47 and Erag to V(D)J recombination in thymocytes, we characterized VEX expression in ETPs from E47- or Erag-null mice.

As shown in Fig. 3 (left), loss of E47 did not significantly alter VEX expression in early triple negative (TN; CD3\(^{-}\) CD4\(^{-}\) CD8\(^{-}\)) thymic progenitors, total CD4\(^{-}\) CD8\(^{-}\) double negative progenitors, or downstream CD4\(^{+}\) CD8\(^{-}\) double positive progenitor subsets. The most noticeable difference occurred in the TN1 cells, in which VEX was slightly reduced from 71% in E47 wild-type mice versus 54% in E47-null mice. Consistent with the minimal effect that loss of E47 has on recombinase activity in early TN subsets, VEX expression was comparable in ETPs from E47-heterozygous versus E47-null mice (not depicted). Likewise, Erag deletion also had little consequence. VEX expression was virtually identical or even slightly elevated in TN1–TN4 subsets from Erag wild-type versus Erag-null mice (Fig. 3, right).

The minimal effect of E47 on VEX expression in T lineage progenitors cannot be attributed to lack of sensitivity of the H2-SVEX recombination substrate because VEX expression completely depends on the presence of \( \text{rag} \) (Fig. S1 A, available at http://www.jem.org/cgi/content/full/jem.20051190/DC1), and recombination efficiency is proportional to levels of \( \text{rag} \) expression (2). Moreover, it is unlikely that the H2-SVEX transgene is subject to significant position-dependent effects because VEX is detectable at comparable levels in hematopoietic tissue from multiple independent founders (2) and is similar for the earliest TN1–TN4 populations across two different founders (compare VEX expression in E47 wild-type H2-SVEX SB110 mice in...
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Fig. 3 to H2-SVEX SB88 mice in Fig. S1 B). These data are consistent with the observation that although E47-null thymic progenitors have altered usage of specific TCR V region genes, these mice do not have a block in TCR recombination (22). Taken together, these data suggest that both E47 and Erg are required for recombinase expression in early B cells but not early T cells.

E47 is required for optimal recombinase activity in DCs and NK cells

We and others have previously shown that the D-J rearrangements at the IgH locus are not B/T lineage–restricted events (2, 23), and we wondered if E47 is required for recombination in other lineages. DC and NK populations have not been characterized in E47-null mice, and we found that these populations are present in reduced numbers. DC precursors in the bone marrow (AA4.1+ CD24hi CD44+ B220+; reference 17) are reduced approximately fivefold from 64,950 ± 31,239 cells (n = 10) in E47 heterozygotes to 11,818 ± 11,096 (n = 10) in E47-null animals (Fig. 2). Similarly, splenic NK cells (NK1.1+ CD122+ CD3–) are reduced threefold from 450,865 ± 217,460 (n = 5) in E47 heterozygotes to 150,434 ± 83,067 (n = 4) from E47-null mice (Fig. 2).

As with pre-pro–B cells, recombinase expression in DC precursors and NK cells is markedly reduced in the absence of E47. VEX expression is decreased from 15 to 4.7% in DC precursors (Fig. 4 A, bottom row) and from 8.9 to 2.8% in NK cells (Fig. 4 B, bottom row) from E47-heterozygous versus E47-null mice, respectively. The observation that recombinase activity is reduced but not ablated in the absence of E47 highlights the potential contribution of other transcription factors to regulate recombination at these stages of development.

These data suggest that the requirements for V(D)J recombination are coordinately regulated in B cells, NK cells, and DCs, and are controlled by distinct mechanisms in the T lineage. This interpretation is consistent with the notion that B and NK cells as well as some DCs develop from bone marrow CLPs, whereas the T lineage appears to most efficiently develop from thymic ETPs (24) in which recombination might be under distinct transcriptional control.

E47 is important for normal development of multipotent lymphoid progenitors

rag expression is first detectable in a rare subset of LSK bone marrow progenitors (1), and recombinase activity is up-regulated in CLPs (2, 24). Specifically, although rag expression is detectable in ~4% of LSKs (1), 40–50% of CLPs have recombination events as measured with the H2-SVEX recombinase substrate (2). Therefore, this developmental transition represents a pivotal stage in dissecting the molecular requirements for V(D)J recombination initiation.

We quantified LSKs and CLPs in E47-null mice to determine how early in development the E47 defect extends. Bone marrow LSKs were detected at comparable frequency (15–22%) in E47 wild-type, E47-heterozygous, and E47-null mice (Fig. 5 A), suggesting that E47 is not required for the generation of LSKs. In terms of absolute cell number, LSKs were somewhat elevated from 56,122 ± 29,989 (n = 3) and 39,530 ± 16,780 (n = 9) in E47 wild-type and E47-heterozygous mice, respectively, to 73,269 ± 81,328 (n = 5) in E47-null mice (Fig. 2). However, these differences were not significant (P > 0.5). Comparable to rag expression (1), recombinase activity is detectable in ~1–1.5% of LSKs (not depicted), and thus, it is difficult to determine whether loss of E47 affects VEX expression at this stage.

In contrast to LSKs, the CLP population was significantly reduced in the absence of E47. We identified bone marrow CLPs using two different phenotypic schemes, again exploiting one staining combination that is independent of the AA4.1 antigen. As shown in Fig. 5 B (top row), for the first phenotypic strategy (17), 17–19% of IL7R+ lin– cells are readily detectable as AA4+ Sca1hi bone marrow CLPs in E47 heterozygotes, but this population is reduced to 0.9–9.9% in E47-null mice shown from three different representative experiments. This 50–95% decrease in the percentage of CLPs is consistent with a 10-fold reduction in absolute cell number in this population from 7,556 ± 3,813 (n = 6) in E47 wild-type mice and 7,842 ± 2,367 (n = 17) in E47-heterozygous mice, respectively, to 724 ± 417 in E47 knockout mice (n = 15; Fig. 2). This residual CLP population in E47-deficient animals also expresses normal levels of c-Kit and only modestly reduced levels of Il3 (Fig. 5 C).

We confirmed the presence of CLPs in E47-null mice using the AA4.1-independent definition IL7R+ lin– Sca1hi c-Kitlo (25). As shown in Fig. 5 D (middle row), these CLPs
are similarly reduced from 19 to 5.2% in E47-heterozygous versus E47-null mice. We then confirmed that AA4.1 is detectable on these CLPs. Interestingly, AA4.1 was expressed on a high percentage of CLPs from E47-null versus E47-heterozygous mice, but it was reduced fourfold in intensity (mean fluorescence intensity: 6,245 vs. 1,494 in E47-heterozygous vs. E47-null mice; Fig. 5 D, bottom row). It is unclear whether this decrease in AA4.1 expression reflects a requirement for E47 for normal levels of expression, or whether AA4.1hi CLPs represent a developmental stage that is absent in the E47-deficient animals.

Early lymphoid progenitors are exquisitely sensitive to apoptosis, and reductions in the CLP compartment could be due to increases in apoptosis or to a failure to proliferate. However, we found that <5% of CLPs or pre-pro–B cells from E47-null animals are annexin V+ DAPI− (Fig. 6 A), consistent with observations that B lymphocyte development in E47-deficient animals cannot be rescued by a bcl2 transgene (26). Although frequencies of apoptotic cells were low, dead pre-pro–B cells (DAPI+) accumulated just over twofold more in E47-null animals (Fig. 6 A and not depicted), possibly reflecting a defect in the ability to clear apoptotic cells. Despite diminished numbers, CLPs and pre-pro–B cells from E47-null mice exhibit twofold higher levels of proliferation compared with E47-heterozygous animals. The percentage of BrdU+ progenitors was increased from 20 to 46% in CLPs and from 37 to 92% in pre-pro–B cells obtained from E47-heterozygous versus E47-deficient animals (Fig. 6 B). Thus,
the reduction in CLPs in E47-null mice is not due to either increased levels of apoptosis or a block in proliferation.

**E47 is indispensable for IgH recombination in multipotent CLPs**

We examined the role of E47 in regulating recombinase activity in AA4.1+ Sca1+ CLPs, an essential step during differentiation to the B lymphocyte lineage. As shown in Fig. 5 B (bottom row), VEX expression is ablated from 36% in CLPs from E47-heterozygous mice to 0% in E47-null mice, suggesting that the activity of E47 is absolutely required for recombinase activity at this stage of development. The complete loss of recombinase activity is consistent across all three species of E47 knockout animals. Not only did CLPs from E47-deficient mice lack recombinase activity, but these progenitors were also deficient in D-JH recombination events at the endogenous IgH locus (Fig. 7 A). D-JH rearrangements were readily detected in CLPs from E47-heterozygous animals but were absent or barely discernable in CLPs derived from E47-null animals using a highly sensitive nested PCR strategy. By quantitative single cell PCR analysis of CLPs, D-JH rearrangements were detectable in 6 out of 26 cells (23%) from E47-heterozygous mice. By contrast, of the more than 50 cells examined in E47-null mice, only one had a D-JH rearrangement (1.9%). Thus, not only are CLPs reduced 10-fold in terms of absolute number in E47-null mice, but the frequency of CLPs capable of undergoing V(D)J recombination is reduced an additional 10-fold.

V(D)J recombination at IgH loci requires rag expression and Ig gene transcription, and E47 has been shown to regulate both processes in cell lines. In primary CLPs, we found that loss of E47 dramatically inhibits rag expression. Consistent with the 100-fold reduction in recombinase-competent lymphoid progenitors, rag1 was not detectable in CLPs from E47-deficient animals despite successful amplification of β-actin transcripts by quantitative RT-PCR (Fig. 7 B). In contrast to the paucity of rag transcripts, μ0 germline transcripts were readily detectable in CLPs from both E47 wild-type and E47-null mice (the frequency of detecting μ0 transcripts in single CLPs was approximately twofold less in E47-null compared with rag1-deficient mice; not depicted).

Taken together, these data indicate that E47 is required for rag1 expression and recombinase activity as early as the CLP stage of development. These results also highlight a previously unrecognized role for E47 in regulating the developmental progression of LSKs to multipotent CLPs.

**DISCUSSION**

The recent definition of a transcriptional hierarchy that controls B lineage development raises fundamental questions about how E47 promotes B lineage specification in multipotent CLPs. Here, we show that E47 controls B lymphocyte production through one effect on CLP development and through a second effect on V(D)J recombination within the CLPs that do develop. These data provide in vivo evidence of a role for E47 influencing lineage commitment in multipotent hematopoietic progenitors and describe the role of E47 as the first essential transcriptional regulator of V(D)J recombinase activity.

Several studies suggested that E2A genes may regulate rag activity or other critical steps in V(D)J recombination, E2A binds to the Erg enhancer (27), and forced expression of E12 or E47 induces rag transcripts in vitro (6, 7). Moreover, whole bone marrow and fetal liver from E2A- or E47-null mice lack detectable rag transcripts and V(D)J recombination events (4, 5). E2A has also been suggested to play a role in regulating IgH chromatin structure (28). However, it was difficult to understand whether E2A gene products control V(D)J recombination in vivo as E2A- and E47-null mice have a complete block in the earliest stages of B lymphopoiesis. Here, we clearly separate the effects of E47 on the development of CLPs and pre-pro-B cells versus the effects of E47 on V(D)J recombination within these subsets.
diminished CLP subset (defined according to two different schemes) is detectable in E47-null mice, and this population lacks rag1 expression, V(D)J recombinase activity, and D-JH recombination events (Figs. 5 and 7). Similarly, a reduced pre-pro–B subset is detectable in E47-null mice, and this population also has significantly reduced levels of recombinase activity (Fig. 1).

Mounting evidence suggests that CLPs are dominantly early B lineage progenitors rather than T lineage progenitors (2, 24). Not only are CLPs much more efficient at giving rise to the B versus T lineage in vivo (29), but our data indicate that V(D)J recombinase activity in CLPs is uniquely sensitive to loss of either E47 (Fig. 5) or the Erag enhancer (2). By contrast, ETPs and other T lineage progenitors appear relatively refractory to E47 or Erag deficiency (Fig. 3 and not depicted). Within the CLP population, the observation that E47-null mice have a more severe defect in V(D)J recombinase activity than Erag-null animals (2) suggests that E47 may regulate rag transcription through Erag-independent targets. Erag only is one of several cis-acting elements that regulate rag (11, 30–32). The D3 enhancer located 8.1 kb upstream of rag2 is of particular interest because D3 controls rag2 expression in B220+ bone marrow cells. D3 contains three potential E-box binding sites, but it remains unknown whether E47 can effectively transactivate this enhancer (31).

The lineage-specific activities of E2A might be controlled by at least three distinct mechanisms. First, E2A activity is antagonized by direct interaction with Ids, proteins that lack a DNA binding domain and therefore act as negative regulators (33, 34). Although relatively little is known about the patterns of Id expression during the earliest stages of hematopoiesis, Id1 is detectable as early as the LSK subset (1), and transgenic mice overexpressing Id1 have significant reductions in B cell development (34). Second, E2A has distinct binding partners in the B versus T lineage that may confer lineage-specific activation potential (35). Third, recent studies tie Notch activity to degradation of E2A protein (36). This observation is interesting because Notch is a key player in the B versus T lineage fate decision, with lower levels of Notch signaling promoting B lymphopoiesis and higher levels of Notch signaling promoting T lymphopoiesis (37, 38). Thus, in a simplified model, low signaling through Notch during progression to the B lineage might be associated with up-regulation of E2A-dependent rag transcription. A wealth of evidence supports a role for both Notch- and Id-dependent regulatory mechanisms at relatively late stages of hematopoietic development, and it would be useful to know whether E47, which is detectable at roughly comparable mRNA levels in rag1-GFP+ LSKs versus rag1-GFP− LSKs (1), has differential functional activity at the protein levels in these subsets.

Our observation that E47 activity is required as early as the CLP stage of development (Figs. 5 and 7) suggests the existence of important, but as yet unidentified, B cell–specific gene targets. The EBF and pax5 transcription factors are two key regulators of B cell development whose activity depends on E47. However, fetal liver progenitors from EBF-null mice retain normal percentages of IL7R+ c-Kit+ hematopoietic progenitors, suggesting that EBF is not essential for the development of this multipotent population (13). Likewise, hematopoietic progenitors from pax5-deficient mice successfully generate D-JH+ pro-B cells, indicating that the pax5 gene product BSAP is also dispensable in CLPs (39). It is also interesting to note that CLPs from E47-null mice have reduced levels of AA4.1 and decreased forward scatter, consistent with a partial block in CLP development. The precise function of AA4.1, a lectin-like type I transmembrane protein (40), remains unknown, and it will be interesting to determine whether up-regulation of AA4.1 expression plays a functional role in lineage progression.

Regulation of IgH chromatin structure by E2A (28) could contribute to lack of V(D)J recombination in E47-deficient mice. However, E47 may not be essential for establishing open chromatin at the IgH locus in CLPs as we detected only a twofold reduction in the frequency of CLPs with germline μ0 transcript. Besides E2A, the related basic helix-loop-helix family members HEB and E2-2 may also be important for progression to the B lymphocyte lineage. Mice that are doubly heterozygous for mutations in any two of these three genes have more profound defects in pro–B cell development than any single heterozygous mutation (41). HEB and E2-2 are detectable in B lineage progenitors from E47-null mice, and low-level activity from these genes in concert with EBF is sufficient to promote reasonably efficient expression of most B lineage–specific genes (14). The different E proteins, including E12, E47, HEB, and E2-2, appear to have different affinity for E-box consensus sites. Relatively little is known about the individual roles that each transcription factor plays during development, although significant redundancies appear to exist. For example, mice expressing a dominant negative allele of HEB that forms nonfunctional heterodimers with E2A show a more pronounced T cell defect than do mice carrying null mutations for either HEB or E2A alone (35). Moreover, E2A complexes can be composed of E12 or E47. E2A is detectable as dominant E47–E12 heterodimers in IL7–expanded pro–B cells, whereas mature splenic B cells contain largely E47 homodimers (42). Whether E2A exists functionally as homodimers or heterodimers in LSKs and CLPs, and whether distinct E protein complexes are can be differentially regulated by Notch– or Id-dependent inhibitory mechanisms, remains to be determined.

Here, we have shown that the activity of E47, a master regulator of B lineage development, is required for expression of both rag1 and V(D)J recombinase activity in multipotent hematopoietic progenitors and B lineage lymphocytes. Distinct factors appear to control recombinase activity in the T lineage. These data provide one molecular mechanism by which E47 promotes B lineage specification in vivo. That additional mechanisms remain to be elucidated is suggested by our finding that E47-deficient mice fail to generate normal numbers of CLPs and pre-pro–B cells.
MATERIALS AND METHODS

Mice. H2-SVEX recombination reporter substrate mice were developed in our laboratory. Two independent H2-SVEX founder lines, SB110 and SB88, have been extensively characterized elsewhere (2) and are inbred C57BL/6 mice. E47−/− mice (43) were crossed to the H2-SVEX background. All mice were treated humanely in accordance with federal and state government guidelines, the University of Massachusetts Medical School institutional animal committee, and the Institutional Animal Care and Use Committee of the University of Pittsburgh.

Flow cytometry. Freshly isolated bone marrow cells or thymocytes were resuspended to 3 × 10^7/ml in staining media containing biotin−, flavin−, and phenol red−deficient RPMI 1640 (Inviren 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 and 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 μg/ml propidium iodide to exclude dead cells. VEX was detected using 407 nm excitation (2). Primary antibodies included thymus AA4.1 biotin or APC; B220 APC or biotin or Cy5PE or FITC; CD3 biotin or Cy5PE; CD4 Cy5PE or PE; CD86 APC or biotin or Cy5PE; CD11b biotin; CD19 biotin or Cy5PE or FITC; CD24 (clone 30-F1) Alexa 594; Cascade Blue or FITC; CD25 Cy7-APC; CD43 PE; CD122 PE; e−K−PE; DX5 biotin or FITC; CD44 FITC; y8−TCR biotin; Gr−1 biotin (clone 8CS); IgM (clone 33B1) biotin or FITC; IL−7RA APC (clone A7R34) or PE (clone SB14); Ly6C biotin or FITC; NK1.1 biotin or FITC; TER−119 biotin; and Sca−1 FITC. Secondary reagents were SA−Cy5PE, SA−Alexa 594, and SA−Cy7PE or SA−APC. Antibodies were purchased from BD Biosciences, ebioscience, Southern Biotechnology Associates, Inc. or Caltag. Annexin V staining was performed according to the manufacturer’s instructions (BD Biosciences) using 2.5 μl annexin V FITC and 0.2 μg/ml DAPI per 1.5 × 10^6 cells. Flow cytometry was performed on a 3 laser, 7 detector DIVA FACSVantage, a 3 laser, 9 detector LSII, or a 3 laser, 10 detector FACS Aria (Becton Dickinson). Data were analyzed with Flowjo software (Tree Star).

BrdU incorporation and annexin staining. For analysis of proliferation, mice were injected ip. with 200 μg BrdU in PBS, or PBS alone as a control, at 12−h intervals as described previously (44). 24 h after the first injection, bone marrow was isolated and cells were stained for surface markers as described above. BrdU detection was performed with the BrdU flow kit (BD Biosciences) according to the manufacturer’s instructions. For analysis of apoptosis, bone marrow populations from unmanipulated mice were stained for surface markers and then incubated with annexin V according to the manufacturer’s instructions, except that DAPI was used instead of propidium iodide (BD Biosciences).

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 (dATP, dGTP, dCTP, and dGTP), 2.5 U Taq, 4 mM MgCl2, 1X Buffer A (Fisher Scientific), and 4 μM of each primer. Primers 2011 (5′) and 2163 (3′) were used to detect the presence of the H2-SVEX (2), whereas E47bas forward (5′) and E47bas reverse (3′) were used to detect E47 (26) under the conditions described in each publication. PCR products were visualized with ethidium bromide on a 1.5% agarose gel in TBE buffer.

Quantitative PCR. Total RNA was prepared from sorted cells using the RNeasy mini kit (Qiagen). The cycle conditions for real-time PCR were 95°C for 12 min followed by 50 cycles at 95°C for 15 s and at 60°C for 1 min. Probes and primer sets for the rag and actin genes were purchased from Applied Biosystems. Real-time PCR was performed in duplicate or triplicate on an ABI Prism 7900HT.

PCR detection of IgH rearrangements. Equal numbers of CLPs from E47-heterozygous or E47-null mice were sorted directly into Eppendorf 96-well plates containing lysis buffer as described previously (21). The PCR protocol involves two rounds of amplification with nested primers. In the first round of PCR, the following three primers were used: GL−5 −1, CCGGACAGAGCCAGGCAAGTGG; D3−5 −1, ACAAGCTCTCAAGACCATGTCTGGCT; and D3−3 −1, AGGCTCTGTAGATCCCTAGAG. In the second round of PCR, two separate reactions were performed using the following primers: germline detection: GL−5 −2, GAGTGTGACTGAGAGGACAG and GL−3 −2, CGAAGTACAGGAGGACAC; and D3−2 rearrangement detection: D3−5 −2, AGGCTCGACTT(G or C)TAAAGGTGATCCTACTGTTG and D3−3 −2, GGCTCTGACTCTCTCAAGGGCCTCCCCTAGGG. In the first round of PCR, amplification was performed in a total volume of 50 μl containing 0.2 mM dNTP, 1 μl CLONTECH Advantage cDNA Polymerase Mix, 1X CLONTECH Advantage PCR Buffer, 0.5 μg/ml BSA, and 0.4 μl of each primer. In the second round, 1 μl of each first-round product was added to a 50-μl reaction volume using the same conditions described above, except that second round primers were used at 2-μM concentrations. The cycling conditions for both rounds were: 95°C for 1 min, 63°C for 1 min, and 72°C for 1.5 min for 30 cycles, with a 10-min end extension at 72°C. PCR productions were visualized on 1.5% agarose gels stained with ethidium bromide. For analysis of single cells, single CLPs were sorted into individual wells and PCR was performed as described above. Only samples producing either a germline product or a specific rearrangement product are included in the analysis.

Statistical analysis. A Wilcoxon rank-sum test was used for statistical comparison of two samples. Multiple comparisons were performed using the Kruskal−Wallis test with Bonferroni correction of p. Differences were regarded as significant at P < 0.05. Analyses were performed using the JMP version 5.1 statistical software package (SAS Institute).

Online supplemental material. Fig. S1 shows that VEX expression in T lineage precursors depends on rag1 and is identical across multiple independent founders (compare Fig. S1 and Fig. 3). Fig. S1 is available at http://www.jem.org/cgi/content/full/jem.20051190/DC1.

We gratefully acknowledge Dave Allman, Avinash Bhandoola, Michelle Kellihier, and Joe Labrie for thoughtful discussions. We thank Kees Murre for providing E47-null mice and Mark Schlissel for providing Ergo-null mice. We also thank Randy Hardy and Ben Rowley for generously sharing protocols and Scott Shell for advice. Kristy Pilbeam, Kiley Maguire, and Heather Paquin provided invaluable assistance with experiments. In addition, we thank Dewanye Fakirer at the University of Pittsburgh Department of Immunology FACS facility as well as the University of Massachusetts Medical School Flow Cytometry Facility for their excellent help.

This work is supported by the American Heart Association (to L. Borghesi), University of Pittsburgh Competitive Medical Research Funding (to L. Borghesi), and National Institutes of Health grant AI043534 (to R. Gerstein).
The authors have no conflicting financial interests.

Submitted: 14 June 2005
Accepted: 2 November 2005

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