

Assembling a Gene Regulatory Network for Specification of the B Cell Fate

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Summary

The generation of B lymphocyte precursors is dependent on the combinatorial action of the transcription factors PU.1, Ikaros, E2A, EBF, and Pax-5. Loss of PU.1 results in a severe reduction in Flk2⁺, IL-7R⁺ lymphoid progenitors as well as impaired expression of EBF and Pax-5. Restoration of EBF expression facilitates rapid generation of pro-B cells from *PU.1*^{-/-} progenitors. Molecular analysis suggests that PU.1 directly participates in regulation of the EBF gene. Although PU.1 is dispensable for expression of most early B lineage genes, it is required for CD45R/B220. Using *EBF*^{-/-} mutant progenitors, we show that EBF induces Pax-5 and the early program of B lineage gene expression. Importantly, Pax-5 does not rescue B cell development from either *PU.1*^{-/-} or *EBF*^{-/-} progenitors. Pax-5 expression and function are contingent on EBF. Based on these results, we propose a hierarchical regulatory network for specification and commitment to the B cell fate.

Introduction

Hematopoietic cell fates appear to be specified by unique combinatorial sets of transcription factors. The B lymphocytic and erythrocytic lineages are leading models for analyzing the regulatory networks that underlie the specification of cell fates. For both lineages, distinct sets of transcription factors have been genetically shown to be required for cell fate specification (Cantor and Orkin, 2002; Schebesta et al., 2002). Cell fate choice in the hematopoietic system is also modulated by various signaling pathways including Wnt, Notch, and cytokines (Allman et al., 2002; Kondo and Weissman, 2000; Reya, 2003). These signaling pathways may establish or modulate regulatory networks by inducing the expression or altering the activity of lineage-determining transcription factors (Dahl et al., 2003; Pui et al., 1999). The characterization of such gene regulatory networks

and their integration with signaling pathways represents a major challenge.

Specification of the B cell fate involves the expression of a set of B lineage-specific genes including mb-1, B29, λ 5, and VpreB and the onset of ordered DNA rearrangements of the immunoglobulin heavy chain (IgH) locus catalyzed by Rag-1 and Rag-2. Early B cell development is critically dependent on five transcriptional regulators, PU.1, Ikaros, E2A, EBF, and Pax-5. Of these, EBF and Pax-5 function exclusively within the B lineage, whereas PU.1, Ikaros, and E2A are important in the development of additional hematopoietic lineages. Both E2A (E12/E47) and EBF are required for initiation of B cell development. Targeted inactivation of the E2A or EBF genes results in a block to B cell development prior to the onset of early B lineage gene expression and initiation of D_H-J_H rearrangements (Bain et al., 1994; Lin and Grosschedl, 1995; Zhuang et al., 1994). E2A, EBF compound heterozygotes are compromised for expression of several early B cell genes, notably Pax-5 (O’Riordan and Grosschedl, 1999). Pax-5 is not required for specification of the B cell fate. *Pax-5*^{-/-} pro-B cells express early B lineage genes and undergo IgH recombination but exhibit extensive developmental plasticity (Nutt et al., 1997, 1999). Conditional inactivation of the Pax-5 gene demonstrates that Pax-5 is continuously required to maintain B lineage commitment (Mikkola et al., 2002). These studies suggest that specification of the B cell fate is dependent on E2A and EBF while commitment and its maintenance require Pax-5.

The functions of PU.1 and Ikaros in B cell development are less well understood. PU.1 is essential for development of both the lymphoid and myeloid lineages, whereas the Ikaros proteins function primarily in the lymphoid lineages (Scott et al., 1994; Wang et al., 1996). PU.1 has been shown to regulate hematopoietic progenitors and their progeny by controlling expression of vital cytokine receptors (DeKoter et al., 1998, 2002). The PU.1 mutation results in a profound block to B lymphopoiesis with an absence of B lineage progenitors expressing early B lineage genes (DeKoter et al., 2002; Scott et al., 1997). *PU.1*^{-/-} multipotential progenitors are defective in expression of the IL-7R α chain. The interleukin-7 receptor (IL-7R) is initially expressed primarily on lymphoid progenitors and is important for the development of B and T lymphocytes (Kondo et al., 1997; Peschon et al., 1994; Sudo et al., 1993). Although it is clear that IL-7R signaling plays a key role in the survival and proliferation of B and T lineage cells, it is not essential for their generation (Carvalho et al., 2001; Crompton et al., 1998). Interestingly, retroviral transduction of the IL-7R α chain in *PU.1*^{-/-} progenitors restored the development of CD19⁺ B lineage precursors (DeKoter et al., 2002). However, the rescue was inefficient, suggesting additional critical functions of PU.1 in B lymphocyte development.

Flk2/Flt3 is a cytokine receptor that is selectively expressed on primitive hematopoietic progenitors and may function in promoting the lymphoid versus myeloid cell fates (Matthews et al., 1991; Rosnet et al., 1991). Upregulation of Flk2/Flt3 on bone marrow multipotential

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progenitors is associated with loss of stem cell and myeloid reconstitution potential *in vivo*, but also with sustained lymphoid potential (Adolfsson et al., 2001). Targeted inactivation of the *flk2/flt3* gene results in a severe deficiency in B lineage precursors (Mackarenhtschian et al., 1995). Furthermore, mice deficient in Flk2/Flt3 ligand (FL) fail to develop normal numbers of IL-7R⁺ lymphoid progenitors (Sitnicka et al., 2002). The combined loss of Flk2/Flt3 and the IL-7R results in a profound block to both fetal and adult B cell development (Sitnicka et al., 2003; Vosshenrich et al., 2003). Interestingly, *PU.1*^{-/-} hematopoietic progenitors have reduced expression of Flk2/Flt3 transcripts (DeKoter et al., 2002).

In addition to the IL-7R and Flk2/Flt3 defects, *PU.1*^{-/-} hematopoietic progenitors are impaired in expression of EBF and Pax-5 (DeKoter et al., 2002). We show that in contrast with the IL-7R α chain, transduction of EBF facilitates the rapid generation of B cell precursors from *PU.1*^{-/-} progenitors. This suggests that the block to B cell differentiation in the *PU.1*^{-/-} embryos is due to impaired expression of the EBF gene. PU.1 binds *in vitro* and *in vivo* to a conserved site in the EBF gene. PU.1, but not EBF, is required for the generation of Flk2/Flt3⁺, IL-7R⁺ lymphoid progenitors and expression of CD45R/B220. EBF functions to induce Pax-5 and the early program of B lineage gene expression. Complementation of *PU.1*^{-/-} and *EBF*^{-/-} progenitors reveals that Pax-5 function is contingent on EBF. Based on these results, we propose a hierarchical regulatory network for specification and commitment to the B cell fate.

Results

EBF Restores Early B Cell Development in *PU.1*^{-/-} Fetal Liver Progenitors

PU.1^{-/-} fetal liver hematopoietic progenitors (Lin⁻ FLPs) are deficient in expression of the *EBF* and *Pax-5* genes. Therefore, we sought to determine if EBF and/or Pax-5 could bypass the requirement for PU.1 in B cell development. Day 14.5 gestation *PU.1*^{+/-} and *PU.1*^{-/-} FLPs were transduced with EBF or Pax-5 encoding retroviral vectors. Following infection, the cells were plated on S17 stromal cells with IL-7. Foci of IL-7-dependent proliferating cells were observed within 4–6 days of transducing EBF. No such cells were observed in Pax-5- or MIGR1-transduced *PU.1*^{-/-} cultures. Transduction of EBF facilitated the generation of IL-7R⁺ cells from the *PU.1*^{-/-} progenitors (Figure 1A). The failure of Pax-5 to rescue the survival or proliferation of *PU.1*^{-/-} FLPs was not due to inefficient expression of Pax-5. The Pax-5 virus expresses at sufficient levels to restore CD19 expression in *Pax-5*^{-/-} pro-B cells (data not shown). These data suggest that EBF can either induce IL-7R expression or rapidly promote the expansion of a rare population of IL-7R⁺ *PU.1*^{-/-} FLPs (see below).

To determine the nature of the cells that were generated by EBF transduction, we harvested day 10–14 MIG-EBF cultures and examined expression of the B lineage marker CD19 and the myeloid marker Mac-1. *PU.1*^{+/-} FLPs transduced with the MIGR1 virus generated CD19⁺ as well as Mac-1⁺ cells, as did *PU.1*^{-/-} FLPs transduced with the MIG-PU.1 virus (data not shown; DeKoter and Singh, 2000). In contrast, the MIG-EBF-transduced cells

were exclusively CD19⁺ (Figure 1B). It was possible that the related Ets family member Spi-B compensated for PU.1 in the EBF-transduced cultures (DeKoter et al., 2002). However, the generation of CD19⁺ IL-7-responsive progenitors was also restored by transduction of EBF in *PU.1*^{-/-} *Spi-B*^{-/-} FLPs (Supplemental Figure S1 at <http://www.developmentalcell.com/cgi/content/full/7/4/607/DC1/>). Thus, EBF can bypass the requirement for PU.1 in early B cell development in the absence of Spi-B.

Next, we addressed if EBF could restore expression of early B lineage genes and IgH recombination in the transduced cells. Since CD19 is a Pax-5 target gene, it was likely that EBF-transduced progenitors expressed Pax-5. Indeed, RT-PCR analysis revealed Pax-5 transcripts in EBF-rescued pro-B cells (Figure 1C). We note that in the PU.1- or EBF-transduced *PU.1*^{-/-} progenitors, expression of EBF and Pax-5 transcripts was restored to near wild-type levels (Supplemental Figure S2). Importantly, the EBF-transduced cells expressed early B lineage genes including mb-1, B29, VpreB, λ 5, Rag-1, and Rag-2 (Figure 1C). Furthermore, these cells expressed robust levels of germline μ transcripts, demonstrating that transcriptional activation of the IgH locus is not dependent on PU.1 (Supplemental Figure S2). To determine if expression of EBF was also sufficient to restore IgH recombination, we performed PCR analysis. D_H-J_H rearrangements were readily observed in EBF-rescued cells after 14 days, whereas both proximal and distal V_H-D_{JH} rearrangements were detectable within 14–21 days (Figure 1D). Thus, EBF can restore B cell differentiation through the pre-B cell stage in *PU.1*^{-/-} FLPs. These results demonstrate that the early program of B cell gene expression is dependent on EBF, but not PU.1. Furthermore, they imply that Pax-5 expression and function are contingent on EBF.

PU.1 Is Required for Expression of CD45R/B220

PU.1 has been suggested to regulate CD45 expression in a lineage-specific manner in myeloid lineage, but not in lymphoid lineage, cells (Anderson et al., 2001). A hallmark of early B lineage development is expression of the CD45 isoform CD45R/B220. Whereas *PU.1*^{-/-} cells rescued with PU.1 expressed CD45R/B220, *PU.1*^{-/-} cells transduced with EBF did not, as revealed by flow cytometry and RT-PCR (Supplemental Figures S3A and S3B). These results demonstrate that PU.1 is required for the expression of CD45R/B220 in B lineage cells. To determine that PU.1 is necessary for expression of CD45 in hematopoietic progenitors, FLPs from *PU.1*^{+/-} and *PU.1*^{-/-} embryos were stained with an antibody that recognizes all forms of CD45 and analyzed by flow cytometry and RT-PCR. As shown in Supplemental Figures S3C and S3D, *PU.1*^{-/-} FLPs lack expression of CD45 protein as well as transcripts. These data demonstrate that expression of CD45 and its isoform CD45R/B220 are dependent on PU.1 in myeloid and B lineage cells.

EBF, but Not IL-7R α , Promotes Rapid Generation of Pro-B Cells from *PU.1*^{-/-} FLPs

The expression of the *IL-7R α* and *EBF* genes is dependent on PU.1. Intriguingly, either IL-7R α or EBF can restore production of CD19⁺ B cell precursors from *PU.1*^{-/-} FLPs *in vitro* (Figure 1B; DeKoter et al., 2002).

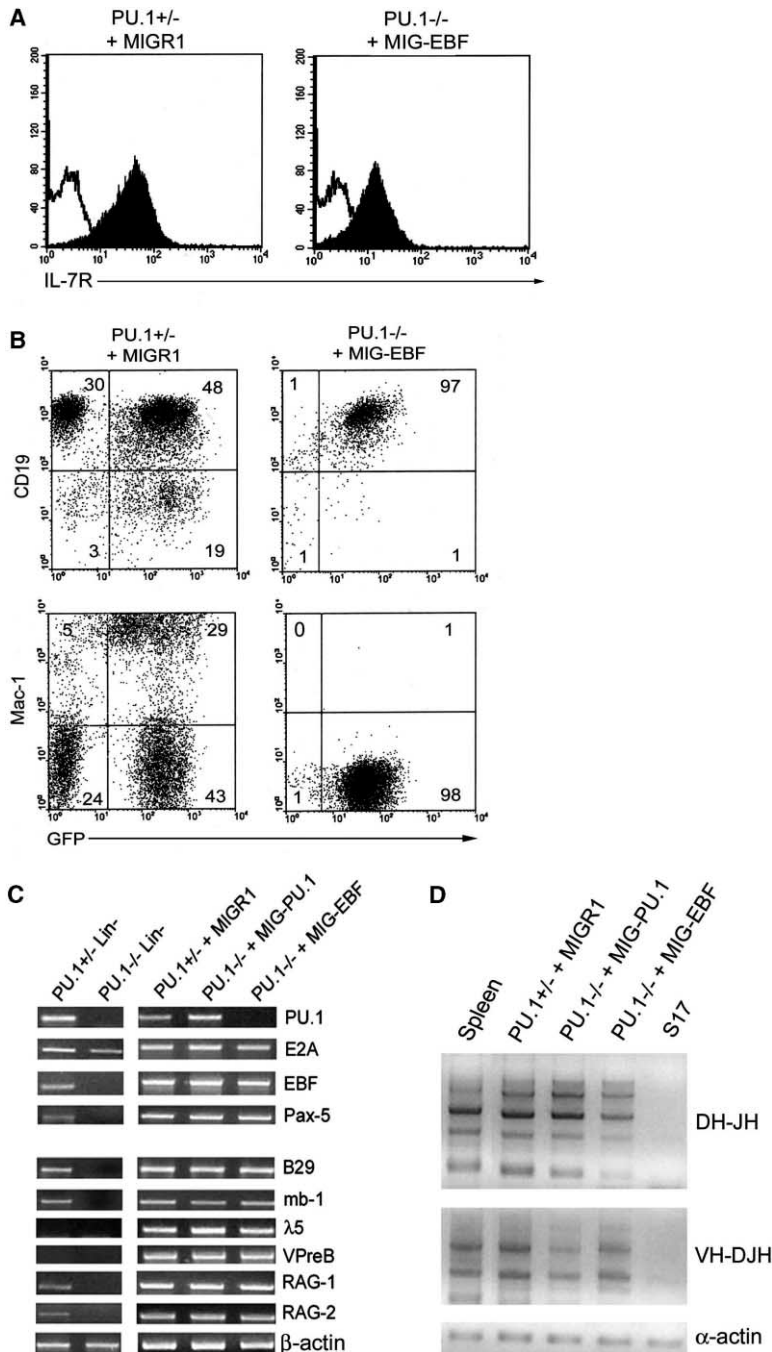


Figure 1. EBF Bypasses the Requirement for PU.1 in Early B Cell Development

(A) IL-7R expression in EBF-transduced progenitors. Comparative analysis of *PU.1*^{+/-} Lin⁻ FLP cells transduced with the MIGR1 virus or *PU.1*^{-/-} Lin⁻ FLP cells transduced with the MIG-EBF virus. Cells were cultured on S17 stromal cells with IL-7 for 10 days and then FACS analyzed. The filled and open histograms represent staining with anti-IL-7R or an isotype control antibody, respectively. (B) Cell cultures described in (A) were analyzed for expression of CD19, Mac-1, and GFP. Quadrants were set based upon staining with appropriate isotype controls.

(C) RT-PCR analysis of B lineage gene expression in Lin⁻ cells isolated from *PU.1*^{+/-} and *PU.1*^{-/-} embryos, *PU.1*^{+/-} Lin⁻ cells transduced with the MIGR1 virus, or *PU.1*^{-/-} Lin⁻ cells transduced with either MIG-PU.1 or MIG-EBF.

(D) Rearrangement of IgH loci in *PU.1*^{-/-} cells transduced with the MIG-PU.1 or MIG-EBF viruses. Genomic DNA from the indicated cells was analyzed by PCR. Similar results were obtained using primers to detect V_H-DJ_H rearrangements involving the 7183 (shown), Q52, or J558 VH families. Data are representative of 3–5 independent experiments.

To compare the ability of IL-7R α and EBF in bypassing the requirement for PU.1 in B cell development, we performed a kinetic analysis. *PU.1*^{-/-} FLPs were transduced with MIG-PU.1, MIG-EBF, or MIG-IL-7R α viruses and the cultures were analyzed periodically for the appearance of B cell precursors (Figure 2). Interestingly, CD19⁺ cells were evident at the earliest time point analyzed in *PU.1*^{-/-} cultures infected with MIG-PU.1 or MIG-EBF viruses. As noted above, foci of proliferating cells were observed in EBF-transduced cultures as early as day 4. In stark contrast, the generation of CD19⁺ cells after transduction with IL-7R α was considerably delayed. We note that at earlier time points, the IL-7R α -transduced

cultures consist exclusively of IL-7-dependent myeloid progenitors (data not shown and DeKoter et al., 2002). Thus, EBF restores the development of B cell precursors more rapidly than the IL-7R. These data suggest that the primary block in B cell differentiation observed in *PU.1*^{-/-} progenitors is due to impaired EBF expression.

PU.1 Binds In Vitro and In Vivo to a Conserved Site in the EBF Gene

To explore if PU.1 directly regulates the *EBF* gene, we pursued a bioinformatics approach. The genomic sequences encoding the mouse and human *EBF* genes

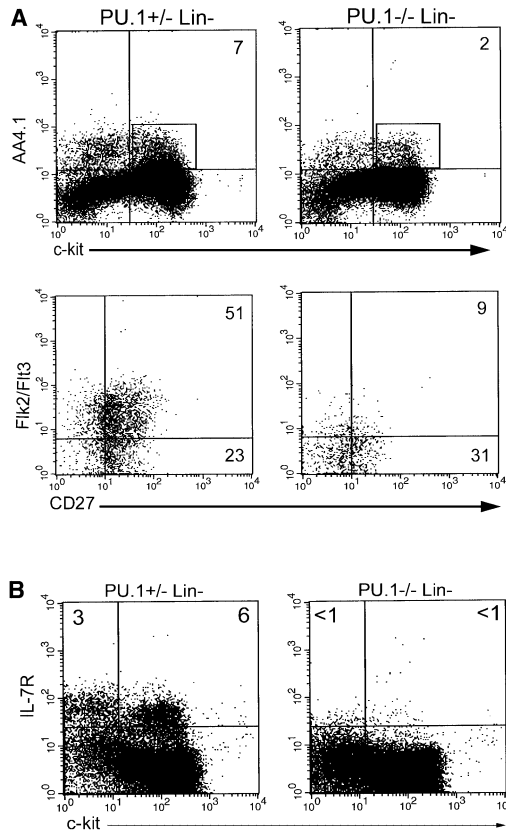


Figure 4. Analysis of Lymphoid Progenitor Compartments in *PU.1*^{+/+} and *PU.1*^{-/-} Embryos

(A) Fetal liver Lin⁻ cells were isolated from *PU.1*^{+/+} and *PU.1*^{-/-} embryos and stained with antibodies to c-kit, AA4.1, CD27, and Fik2/Flt3. The c-kit⁺ AA4.1⁺ cells (boxed regions in upper panels) were analyzed for expression of CD27 and Fik2/Flt3 (lower panels). Lymphoid progenitors are a subset of the c-kit⁺ AA4.1⁺ multipotential cells that express both CD27 and Fik2/Flt3. Data are representative of three independent experiments.

(B) Fetal liver Lin⁻ cells were stained with antibodies to c-kit and IL-7R. Data are representative of two independent experiments. Quadrants were set based upon staining with appropriate isotype controls.

combination was designed to resolve various multipotential progenitors (Cumano et al., 1992; Matthews et al., 1991; Medina et al., 2001; Phillips et al., 2000). Initially, we compared frequencies of Lin⁻ c-kit⁺ AA4.1⁺ progenitors and found a 3-fold reduction in *PU.1*^{-/-} compared to *PU.1*^{+/+} embryos (Figure 4A, 2% ± 1% versus 7% ± 1%, respectively; Scott et al., 1997). Next, we examined percentages of Lin⁻ c-kit⁺ AA4.1⁺ cells expressing CD27 and Fik2/Flt3. In *PU.1*^{+/+} fetal livers, 53% ± 2% of Lin⁻ c-kit⁺ AA4.1⁺ cells expressed CD27 and Fik2/Flt3 (Figure 4A, bottom left). In contrast, only 12% ± 4% of Lin⁻ c-kit⁺ AA4.1⁺ cells in *PU.1*^{-/-} fetal liver expressed CD27 and Fik2/Flt3 (Figure 4A, bottom right). Taken together, these data reveal a significant decrease in Lin⁻ c-kit⁺ AA4.1⁺ CD27⁺ Fik2/Flt3⁺ progenitors in *PU.1*^{-/-} embryos compared to their wild-type counterparts. Furthermore, the *PU.1*^{-/-} c-kit⁺ AA4.1⁺ CD27⁺ cells expressed lower levels of the Fik2/Flt3 receptor. As a likely consequence, the frequency of c-kit⁺

IL-7R⁺ progenitors is severely diminished in *PU.1*^{-/-} embryos (Figure 4B).

To determine if Fik2/Flt3 expression on FLPs correlated with enhanced B cell potential, we performed limiting dilution analysis. The B lineage precursor frequencies of c-kit⁺ AA4.1⁺ Fik2⁺ and c-kit⁺ AA4.1⁺ Fik2⁻ FLPs were compared. Using this assay, we observed a 4-fold increase in B cell precursor frequency in c-kit⁺ AA4.1⁺ cells expressing the Fik2/Flt3 receptor (Supplemental Figure S4). In contrast, myeloid developmental potential was increased only 2-fold in the Fik2⁺ compared to the Fik2⁻ fraction (Supplemental Figure S4). Thus, acquisition of Fik2 expression correlates with enhanced B cell precursor activity in fetal liver. Together with the data presented above, these data suggest a profound block in the development of lymphoid progenitors in *PU.1*^{-/-} embryos prior to IL-7R expression.

Next, we determined if the impaired development of Fik2⁺, IL-7R⁺ lymphoid progenitors in *PU.1*^{-/-} embryos was due to lack of FL production by multipotential progenitors. Using RT-PCR analysis, we found no defect in expression of FL in *PU.1*^{-/-} FLPs (Supplemental Figure S5). As expected, Fik2/Flt3 transcripts were diminished, consistent with the flow cytometry data. These results suggest that the impaired expression of Fik2/Flt3 receptor, rather than its ligand, contributes to the B lymphoid deficiency in *PU.1*^{-/-} embryos. The 5-fold reduction in the frequency of *PU.1*^{-/-} FLPs that could be rescued by EBF or IL-7Rα compared with PU.1 (Supplemental Table S1) is correlated with the reduction in the Fik2/Flt3⁺ compartment in *PU.1*^{-/-} embryos (Figure 4A). It is therefore likely that EBF induces B cell differentiation from this rare population of mutant progenitors.

EBF Functions in B220⁺ IL-7R⁺ Lymphoid Progenitors to Specify the B Cell Fate

We have established that PU.1 is needed for the generation of Fik2⁺, IL-7R⁺ lymphoid progenitors (Figure 4). To determine if defective EBF expression contributes to the loss of these progenitors in *PU.1*^{-/-} embryos, we examined the effect of the EBF mutation on the development of such cells. Percentages of Fik2⁺, IL-7Rα⁺ cells were essentially identical between wild-type and *EBF*^{-/-} embryos (Figures 5A and 5B). Therefore, the deficiency of lymphoid progenitors in *PU.1*^{-/-} embryos is not due to impaired expression of EBF. Furthermore, this data rigorously distinguishes the effect of the *PU.1* mutation from that of the *EBF* mutation in the lymphoid developmental compartment. Early B cell progenitors express CD45R/B220. *EBF*^{-/-} embryos (day 14.5 gestation) have B220⁺ progenitors at frequencies comparable to their wild-type counterparts (1.9% ± 0.9% versus 1.8% ± 0.4%, respectively, n = 5 for each genotype). Given the presence of IL-7R⁺ B220⁺ progenitors in *EBF*^{-/-} embryos, we tested if they could be propagated on stromal cells in the presence of IL-7 and stem cell factor (SCF). *EBF*^{-/-} cells readily proliferated in IL-7 and SCF. As shown in Figure 6A, the *EBF*^{-/-} progenitors were CD45R/B220⁺ but did not express CD19. Thus, IL-7-responsive CD45R/B220⁺ cells can be readily expanded from *EBF*^{-/-} FLPs. This contrasts with the behavior of *PU.1*^{-/-} FLPs and is consistent with the view that PU.1 functions upstream of EBF to generate IL-7-responsive lymphoid progenitors.

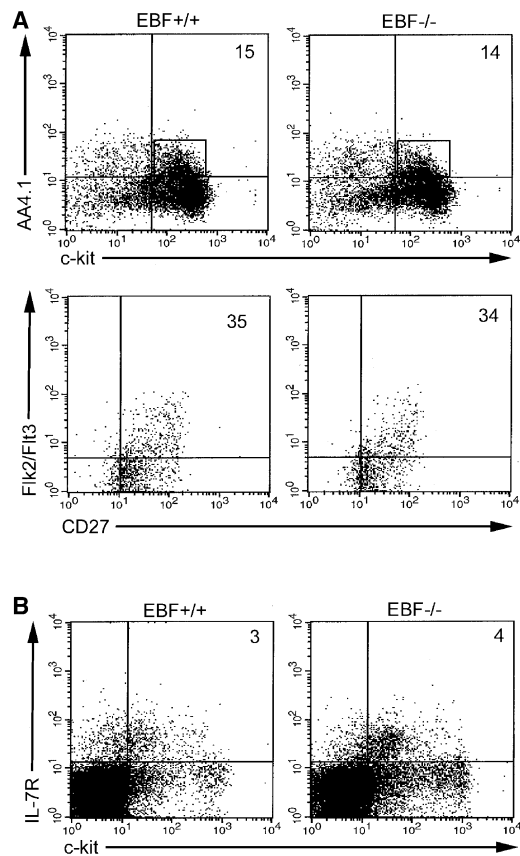


Figure 5. Analysis of the Lymphoid Progenitor Compartment in *EBF*^{-/-} Embryos

(A) Lin⁻ cells from *EBF*^{+/+} or *EBF*^{-/-} embryos were stained with antibodies to c-kit, AA4.1, CD27, and Flk2/Flt3. The c-kit⁺ AA4.1⁺ cells (boxed regions in upper panels) were analyzed for expression of CD27 and Flk2/Flt3 (lower panels). Lymphoid progenitors are a subset of the c-kit⁺ AA4.1⁺ multipotential cells that express both CD27 and Flk2/Flt3. Data are representative of two wild-type and three *EBF*^{-/-} embryos.

(B) Fetal liver mononuclear cells from wild-type or *EBF*^{-/-} embryos were stained with antibodies to c-kit and IL-7R. Data are representative of two wild-type and three *EBF*^{-/-} embryos.

We next determined if restoration of EBF expression in *EBF*^{-/-} progenitors could induce the generation of B cell precursors. FLPs from *EBF*^{-/-} embryos were transduced with the MIGR1 or MIG-EBF retroviral constructs. Transduction of EBF in the mutant progenitors induced expression of CD19 (Figure 6B). Therefore, it was likely that EBF-rescued progenitors expressed Pax-5. As shown in Figure 6C, RT-PCR analysis revealed transcripts for Pax-5 and early B lineage genes. In contrast, MIGR1-transduced *EBF*^{-/-} progenitors lacked expression of Pax-5 and most early B lineage genes. Low levels of B29 transcripts were detectable in the MIGR1-transduced *EBF*^{-/-} cells. Importantly, *EBF*^{-/-} cells expressed normal levels of PU.1, E2A, and IL-7R α transcripts. Finally, we note that transduction of Pax-5 in *EBF*^{-/-} progenitors did not induce expression of CD19 in a significant fraction of cells (Figures 6B and 6D) nor bypass the requirement for EBF in induction of mb-1 expression (Figure 6D). Taken together, these results establish that induction of early B lineage gene expression in lymphoid

progenitors requires EBF. Furthermore, they demonstrate that Pax-5 expression and function are contingent on EBF.

Discussion

Using *PU.1*^{-/-} or *EBF*^{-/-} FLPs, we have attempted to delineate the roles of the transcription factors PU.1, EBF, and Pax-5 in the development of B cell precursors. *PU.1*^{-/-} FLPs are defective in expression of EBF and Pax-5, and herein we demonstrate that EBF can bypass the requirement for PU.1 in early B cell development. In addition, we provide evidence that PU.1 can participate directly in regulating expression of the *EBF* gene. Importantly, we show that Pax-5 expression and function are contingent on EBF. We also provide evidence for a unique function of PU.1 in development of the lymphoid progenitor compartment that cannot be executed by EBF or the combinatorial action of EBF, E2A, and Ikaros. Finally, by complementing *EBF*^{-/-} progenitors, we establish that EBF, but not Pax-5, is required for specification of the B cell fate. These results lead us to propose a gene regulatory network that orchestrates the development of B lineage precursors.

PU.1, but not EBF, is important for development of the c-kit⁺ AA4.1⁺ CD27⁺ Flk2/Flt3⁺ compartment from which fetal liver B lineage progenitors are generated (Figures 4A and 5A). This analysis rigorously resolves the functions of these two transcription factors in the multipotential progenitor compartment. Multipotential progenitors expressing Flk2/Flt3 are enriched for B lineage developmental potential (Supplemental Figure S4), and in vitro data suggests that ligation of Flk2/Flt3 promotes IL-7R α expression (Adolfsson et al., 2001; Borge et al., 1999). B, but not myeloid lineage deficiencies, have been found in both Flk2/Flt3 and Flk ligand (FL) knockout animals (Mackarehntschian et al., 1995; Sitnicka et al., 2002). The combined loss of Flk2/Flt3 and IL-7R signaling results in failure to develop fetal as well as adult B cell precursors (Sitnicka et al., 2003; Vosshenrich et al., 2003). Importantly, both Flk2/Flt3 and IL-7R α transcripts are severely reduced in *PU.1*^{-/-} FLPs (Supplemental Figure S5; DeKoter et al., 2002). We now show a significant reduction in c-kit⁺ AA4.1⁺ CD27⁺ Flk2/Flt3⁺ progenitors in *PU.1*^{-/-} embryos, and those that are detectable express lower levels of the Flk2/Flt3 receptor (Figure 4B). The reduction in Flk2/Flt3⁺ progenitors is not due to defective production of FL (Supplemental Figure S5). These results suggest that PU.1 may regulate the expression of the Flk2/Flt3 gene and thereby the survival/proliferation of Flk2/Flt3⁺ lymphoid progenitors. Expression of Flk2/Flt3 is also dependent on Ikaros. Flk2/Flt3 mRNA is absent in both *Ikaros* null and *Ikaros DN*^{-/-} Lin⁻ hematopoietic progenitors (Nichogiannopoulou et al., 1999). Since Ikaros expression is not impaired by the PU.1 mutation, we propose that expression of Flk2/Flt3 may depend on concerted regulation by PU.1 and Ikaros (see model, Figure 7). Interestingly, the decreased frequency of Flk2/Flt3⁺ lymphoid progenitors in *PU.1*^{-/-} embryos (Figure 4A) correlates with our clonal analysis comparing the efficiency of EBF and PU.1 rescue (Supplemental Table S1). Thus, EBF may be restoring B cell development from the *PU.1*^{-/-} c-kit⁺ AA4.1⁺ CD27⁺ Flk2/Flt3⁺ population. In contrast, PU.1 could function in a less restricted

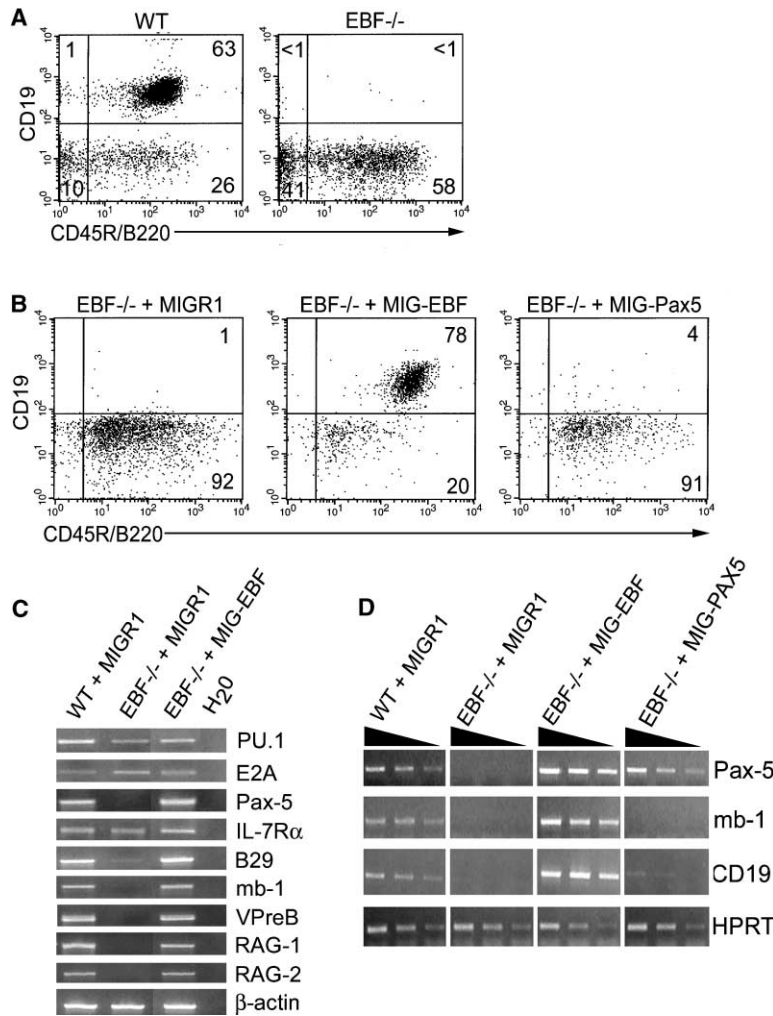


Figure 6. EBF but Not Pax-5 Restores B Cell Differentiation in *EBF*^{-/-} FLPs

(A) Lin⁻ cells from *EBF*^{+/+} and *EBF*^{-/-} embryos were isolated and plated on S17 stromal cells in medium containing IL-7 and SCF. Day 10–14 cultures were harvested and analyzed for expression of CD45R/B220 and CD19.

(B) Comparative analysis of *EBF*^{-/-} Lin⁻ cells transduced with MIGR1, MIG-EBF, and MIG-Pax5 viruses. Transduced cultures were harvested after 10–14 days and analyzed for expression of GFP, CD45R/220, and CD19. The dotplots shown are gated on GFP⁺ cells. Data in (A) and (B) are representative of four independent experiments.

(C) RT-PCR analysis of B lineage gene expression in *EBF*^{-/-} progenitors after transduction with the MIGR1 or MIG-EBF viruses. Data are representative of three independent experiments. Wild-type Lin⁻ cells infected with the MIGR1 virus and differentiated into pro-B cells were used as a control.

(D) Semiquantitative RT-PCR analysis of expression of Pax-5 target genes in *EBF*^{-/-} cultured fetal liver progenitors transduced with the MIGR1, MIG-EBF, or MIG-PAX5 retroviral constructs. Wild-type Lin⁻ cells infected with the MIGR1 virus and differentiated into pro-B cells were used as a control. Data are representative of results obtained after transduction of three independent *EBF*^{-/-} lines.

progenitor population to induce Flk2/Flt3 and IL-7R expression, thereby restoring the lymphoid progenitor compartment and subsequently EBF-dependent B cell differentiation (Figure 7). Based on these considerations, PU.1 would be predicted to be more efficient at a clonal level in restoring B cell development from *PU.1*^{-/-} FLPs than EBF (Supplemental Table S1).

Transduction of EBF in *PU.1*^{-/-} FLPs facilitates IL-7-dependent proliferation, early B lineage gene expression, and IgH recombination, hallmarks of specified

pro-B cells (Figure 1). EBF and E2A synergistically activate transcription of early B lineage genes (O’Riordan and Grosschedl, 1999; Sigvardsson et al., 1997). Furthermore, EBF and E2A can induce specific DNA rearrangements of Ig loci when ectopically expressed with Rag-1 and Rag-2 in nonlymphoid cells (Romanow et al., 2000). E2A has been implicated in regulating EBF expression (Kee and Murre, 1998). The EBF promoter contains a functional binding site for E2A (Smith et al., 2002). Recently, it was shown that *E47*^{-/-} FLPs do not express

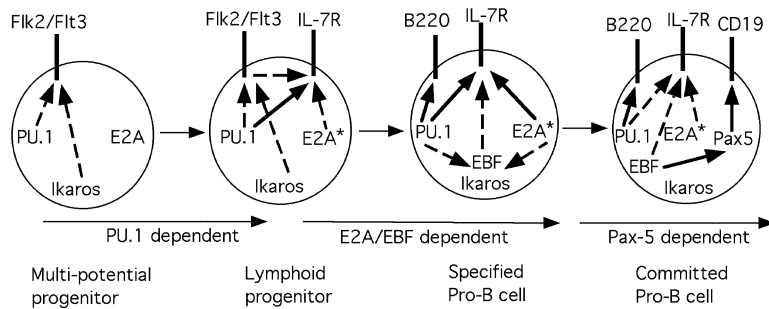


Figure 7. A Hierarchical Regulatory Network for Specification of the B Cell Fate

Four key developmental states are depicted. Each state is dictated by a distinctive combination of regulatory molecules (transcription factors and receptors). Regulatory connections (hatched or solid arrows) are based on experimental evidence presented herein or described in the literature (see Discussion). The cell surface phenotypes of the multipotential progenitor, the lymphoid progenitor, the specified pro-B cell, and the committed pro-B cell are CD45⁺ c-kit⁺ CD27⁺ AA4.1⁺ Flk2⁺, CD45⁺ c-kit⁺ CD27⁺ AA4.1⁺ Flk2⁺ IL-7R⁺, CD45R⁺ IL-7R⁺, and CD45R⁺ IL-7R⁺ CD19⁺, respectively. The specified pro-B cell expresses B29, mb-1, λ 5, VpreB, and Rag1/2.

EBF and restoration of EBF expression promotes the generation of B cell precursors (Seet et al., 2004). These results support the contention that expression of the *EBF* gene is regulated by E2A proteins. Furthermore, they suggest that EBF can function synergistically with low levels of alternative E proteins to regulate the early B lineage program of gene expression. We have characterized a high-affinity PU.1 binding site in the *EBF* gene that is bound both in vitro and in vivo in a pro-B cell line (Figure 3). This site is located within the first intron in a conserved region between the mouse and human *EBF* genes. The conserved region may represent an enhancer or an alternative *EBF* promoter since the translational initiation codon resides in exon 2. Functional analyses are being pursued to test these possibilities. It remains possible that expression of the *EBF* gene is indirectly regulated by PU.1. Nevertheless, the current results lead us to propose that the developmental induction of *EBF* expression depends on the concerted action of PU.1 and E2A (Figure 7). Once expressed, EBF together with E2A then functions to specify the B cell fate by coordinately inducing the early program of B cell gene expression.

Our study reveals CD45R/B220 as a likely PU.1 target gene in B lineage progenitors. *PU.1*^{-/-} CD19⁺ cells rescued with EBF lack expression of CD45R/B220, whereas PU.1-rescued progenitors express CD45R/B220 (Supplemental Figure S3A). A binding site for PU.1 has been characterized upstream of the major transcription start site utilized in myeloid and lymphoid cells (Anderson et al., 2001). However, in this report, PU.1 was suggested to regulate CD45 expression in the myeloid but not lymphoid compartments. In contrast, our results establish that expression of CD45 as well as CD45R/B220 in both the B and myeloid lineages is dependent on PU.1 (Supplemental Figure S3).

In *EBF*^{-/-} bone marrow, B cell development is arrested prior to the expression of B lineage genes and the onset of IgH recombination (Lin and Grosschedl, 1995). Molecular studies have implicated EBF in multiple aspects of B cell development including regulation of Pax-5 and early B lineage gene expression, mediating accessibility of Ig recombination signals, and preventing heterochromatin assembly (Lin and Grosschedl, 1995; Lundgren et al., 2000; Romanow et al., 2000). We show in *EBF*^{-/-} embryos that IL-7R⁺ B220⁺ cells are detectable at wild-type frequency and can be propagated in vitro and differentiated into B cell precursors upon complementation with EBF. The IL-7R⁺ CD45R/B220⁺ lymphoid progenitors expanded from *EBF*^{-/-} embryos express normal levels of PU.1 and E2A transcripts as well as low levels of B29, but do not express Pax-5, mb-1, λ5, VpreB, or Rag-1/2 (Figure 6). Transduction of EBF in the *EBF*^{-/-} progenitors induces expression of all essential early B lineage genes including CD19 and IgH recombination (Figure 6 and data not shown). Interestingly, Pax-5 was unable to induce efficient expression of CD19 or mb-1 in *EBF*^{-/-} progenitors, suggesting that Pax-5 function in activating these two genes is contingent on EBF. This model system employing *EBF*^{-/-} progenitors will facilitate a molecular analysis of the regulatory circuitry used in specification of the B cell fate.

We demonstrate that EBF can restore expression of the *Pax-5* gene, consistent with a previous report of

functional EBF binding sites in the *Pax-5* promoter (Figures 1C and 6C; O’Riordan and Grosschedl, 1999). However, Pax-5 does not appear to reciprocally regulate expression of the *EBF* gene. Deletion of Pax-5 in pro-B cells does not impair EBF expression (Souabni et al., 2002). In our experiments, Pax-5 was unable to rescue B cell development from *PU.1*^{-/-} progenitors. Consistent with our results, EBF, but not Pax-5, could promote the generation of B cell precursors from *E47*^{-/-} FLPs (Seet et al., 2004). Thus, Pax-5 function is contingent on EBF. Furthermore, Pax-5 cannot induce EBF in either *PU.1*^{-/-} or *E47*^{-/-} FLPs. Recently, it has been proposed that Pax-5 can positively regulate the *EBF* gene (Fuxa et al., 2004). In these experiments, precocious Pax-5 expression in the hematopoietic system resulted in thymocytes that express EBF. However, the developmental and molecular basis of this finding remains to be explored.

It is intriguing that either a key transcriptional regulator (EBF) or a cytokine receptor (IL-7R) can facilitate the proliferation and differentiation of *PU.1*^{-/-} progenitors into B lineage precursors, albeit with very different kinetics. Both EBF and IL-7R likely promote the generation of B cell precursors from the same diminished *PU.1*^{-/-} multipotential progenitor population (c-kit⁺ AA4.1⁺ CD27⁺ Flk2/Flt3⁺; Figure 4). The transduced cells exhibit a similar B lineage differentiation program. In both cases, the CD19⁺ B cell precursors do not express CD45R/B220. We suggest that in the case of IL-7Rα transduction, IL-7R signaling promotes the survival/proliferation of rare *PU.1*^{-/-} lymphoid progenitors and kinetically delayed induction of EBF that then results in their differentiation into CD19⁺ B cell precursors. We note that *PU.1*^{-/-} progenitors, if cultured directly on stroma with IL-7 and SCF, can also give rise at low frequency and with delayed kinetics to B220⁻ CD19⁺ B cell precursors (M. Ye and T. Graf, personal communication and data not shown). On the other hand, EBF transduction in *PU.1*^{-/-} lymphoid progenitors induces B cell development without a kinetic delay. EBF likely promotes IL-7R expression in concert with E2A. These results suggest two independent modes of regulation (PU.1- or EBF-dependent) of the IL-7Rα gene. Thus, a key function of PU.1 is to control the generation of lymphoid progenitors. In its absence, these progenitors are severely diminished, likely due to defective cytokine receptor expression (Flk2/Flt3 and IL-7Rα). Furthermore, based on our results, we propose that *PU.1*^{-/-} lymphoid progenitors are defective in undergoing B cell differentiation because of impaired expression of the *EBF* gene. These results illustrate similarities and differences in the role of PU.1 in the development of macrophages versus B lymphocytes. In the myeloid compartment, PU.1 is required not only for the generation of myeloid progenitors, but also for their differentiation into macrophages (DeKoter et al., 1998). In the lymphoid compartment, PU.1 functions to generate lymphoid progenitors but is not directly required for their differentiation. The early program of B cell-specific gene expression is largely independent of PU.1 and Spi-B and is promoted by E2A, EBF, and Pax-5.

Based on previous results and our new findings, we propose a hierarchical regulatory circuitry for specification of the B cell fate (Figure 7). In our model, PU.1 and Ikaros are suggested to induce the expression of Flk2/

Flt3⁺ in primitive hematopoietic progenitors. Signaling through Flk2/Flt3 promotes lymphoid specification and induction of IL-7R in a PU.1-dependent manner. Within the Flk2/Flt3⁺ lymphoid progenitor, PU.1 and E2A cooperate to activate EBF expression. We note that in *PU.1* mutants, few multipotential progenitors express or achieve adequate levels of Flk2/Flt3 to signal expansion/survival of the lymphoid progenitor pool, nor are they able to effectively induce EBF expression. In conjunction with PU.1, an alteration in E2A activity is likely critical for EBF expression and all subsequent events. The mechanism by which this alteration is achieved remains to be uncovered. It has been suggested that E2A activity (denoted as E2A*) is held in check in hematopoietic progenitors by dimerization with inhibitory bHLH proteins including Id family members and Scl/Tal-1, as well as by Notch signaling (Allman et al., 2002; Massari and Murre, 2000). Once expressed at adequate levels, EBF acting in concert with E2A would activate the transcription of early B lineage genes as well as promote IgH recombination resulting in specification of the B cell fate. The final step in the hierarchical regulatory network would involve activation of Pax-5 expression by EBF and E2A. This would culminate in commitment to the B cell fate.

Experimental Procedures

Construction of Retroviral Vectors and Production of Viral Supernatants

The MIGR1 and MIG-PU.1 retroviral vectors have been described (DeKoter et al., 2002; Pear et al., 1998). The EBF and Pax-5 vectors are derivatives of MIGR1. Retroviral supernatants were generated by calcium phosphate transfection of the packaging cell line Plat-E and titered using NIH-3T3 cells (Morita et al., 2000; Walsh et al., 2002). Equivalent titers were used for infection. GP+E-86 packaging cells were used to generate MIG-EBF and MIG-Pax5 viral producer lines (DeKoter et al., 2002).

Isolation and Retroviral Infection of Lin⁻ FLPs

Timed matings between wild-type, *PU.1*^{+/-}, *PU.1*^{+/-}*Spi-B*^{-/-}, and *EBF*^{+/-} mice were performed and embryos obtained at day 14.5 gestation. *PU.1*^{-/-} or *PU.1*^{-/-}*Spi-B*^{-/-} embryos were identified by flow cytometry and confirmed by genotyping (DeKoter et al., 2002). *EBF*^{-/-} embryos were identified by genotyping using the following primers: forward, GGA AAAAGTTGCCTTGAAGTTG; reverse, TGTAGA GGAGCTGGAGCCG; and neo, GCGATGCCTGCTTGCCGAA. Lin⁻ FLPs were isolated as before with the exception that anti-CD19 was included (DeKoter et al., 2002). *PU.1*^{+/-} and *PU.1*^{-/-} FLPs were suspended in retroviral supernatants with 12.5 μg/ml polybrene and centrifuged at room temperature, 3100 rpm for 2.5 hr. After recovery at 37°C, the cells were washed and plated on irradiated S17 stromal cells with IL-7 (5 ng/ml). The cultures were maintained for 10–14 days before analysis. In experiments where the efficiency of PU.1, EBF, and IL-7Rα rescue were compared, the cultures were analyzed at days 10 and 14, and then nonadherent cells were transferred to fresh S17 stroma every 7 days. *EBF*^{+/+} or *EBF*^{-/-} FLPs were infected by coculture with retroviral producing GP+E-86 lines. After infection, the cells were propagated on S17 stroma with IL-7 (5 ng/ml).

Antibodies and Flow Cytometry

Cell suspensions were generated from *PU.1*^{+/-} or *PU.1*^{-/-} fetal livers, and flow cytometry performed as before (Medina et al., 2000). Cells were analyzed on a FACSCalibur using CellQuest software. The antibodies used were: AA4.1 FITC, IL-7R PE or biotin, c-kit APC, CD135/Flk2/Flt3 PE, CD27 biotin, CD11b/Mac-1 PE, CD19 PE or biotin, CD45 biotin, and CD45R/B220 biotin. Biotinylated antibodies were detected with streptavidin-PE-Cy7 (Caltag). All antibodies were purchased from Pharmingen with the exception of AA4.1 FITC.

Limiting Dilution Analysis

After spin-infection, *PU.1*^{+/-} and *PU.1*^{-/-} FLPs were plated at varying dilutions on S17 stroma with IL-7 and analyzed after 10–14 days of culture. For analysis of B cell precursor frequency of c-kit⁺ AA4.1⁺ Flk2⁺ and c-kit⁺ AA4.1⁺ Flk2⁻ populations, 5–200 cells were sorted directly into the wells of plates containing 10⁴ irradiated S17 cells in medium supplemented with IL-7 (5 ng/ml), SCF (10 ng/ml), and FL (50 ng/ml). Six wells were analyzed with each cell concentration for production of CD19⁺ cells after 8 days.

Culture of *EBF*^{-/-} Lymphoid Progenitors

EBF^{-/-} lymphoid progenitors were propagated by plating FLPs from *EBF*^{-/-} day 14.5 embryos on irradiated S17 stromal cells in Optimem medium (GIBCO-BRL) supplemented with 5% fetal calf serum, IL-7 (5 ng/ml), and SCF (10 ng/ml).

RT-PCR Analysis

Total RNA was isolated and converted into cDNA as described (DeKoter et al., 2002). All RT-PCR primers have been published except: EBF forward, GCCTTCTAACCTGCGGAAATC and reverse, GGCGCACATAGAAATCCTGTG; FL forward, CCAGCCTGGAGCC CAAATTC and reverse, GGGCAGCAGGTGGAGGAGTC; CD45/CD45R, forward, CACTGTGTATCTTGCCAAAC and reverse, AGGCG TGAGTGTGGGATTGTC.

PCR Analysis of IgH Rearrangement

PCR using genomic DNA was performed as described before (Medina et al., 2000). The primers used were α-actin (Hardy et al., 1991), DHL (5') (Schlissel et al., 1991), JH4A (3') (Ehlich et al., 1994), and 7183, Q52, and J558 (5') (Schlissel et al., 1991).

Gel Shift Assays

Nuclear extracts were made from 38B9 pro-B cells and gel shift assays were performed as described (DeKoter et al., 2002).

Chromatin Immunoprecipitation Assays

Chromatin immunoprecipitation assays were performed as described with minor modifications (DeKoter et al., 2002). The EBF gene primers are forward, TGGCCAGGCTCTGTGAACA and reverse, TCTTCCCTTCCGTGCGTGTA.

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Note Added in Proof

We note that Maier et al. (Maier, H., Ostraat, R., Gao, H., Fields, S., Shinton, S.A., Medina, K.L., Ikawa, T., Murre, C., Singh, H., Hardy, R.R., and Hagman, J. (2004). Early B cell factor cooperates with Runx1 and mediates epigenetic changes associated with *mb-1* transcription. *Nat. Immunol.*, in press. Published online September 12, 2004. 10.1038/ni1119) have uncovered a molecular mechanism regulating *mb-1* gene transcription, which is consistent with the hierarchical dependence of Pax-5 function on EBF, demonstrated herein.