Immunity, Vol. 11, 21–31, July, 1999, Copyright ©1999 by Cell Press

Coordinate Regulation of B Cell Differentiation by the Transcription Factors EBF and E2A

Mary O'Riordan and Rudolf Grosschedl*†
Howard Hughes Medical Institute
Department of Microbiology and Immunology
University of California, San Francisco
San Francisco, California 94143

Summary

The transcription factors EBF and E2A are required at a similar step in early B cell differentiation. EBF and E2A synergistically upregulate transcription of endogenous B cell–specific genes in a non-B cell line. Here, we examine a genetic collaboration between these factors in regulating B lymphopoiesis. We find that *Ebf*$^{+/-}$*E2a*$^{+/-}$ mice display a marked defect in pro-B cell differentiation at a stage later than observed in the single homozygous mutant mice. Pro-B cells from *Ebf*$^{+/-}$*E2a*$^{+/-}$ mice show reduced expression of lymphoid-specific transcripts, including *Pax5*, *Rag1*, *Rag2*, and *mb-1*. We also show that EBF directly binds and activates the *Pax5* promoter. Together, these data show collaboration between EBF and E2A and provide insight into the hierarchy of transcription factors that regulate B lymphocyte differentiation.

Introduction

The differentiation of B lymphocytes from hematopoietic progenitors is a complex and strictly regulated process. Stages of early B cell differentiation have been characterized by sequential rearrangement of the immunoglobulin (Ig) genes and by the ordered appearance or disappearance of proteins at the cell surface (Rolink and Melchers, 1993). This program of differentiation involves the selective expression of genes that are characteristic of the B cell lineage. The correct temporal expression of these cell type-specific genes is determined by the action of lineage-restricted transcriptional regulators. Experiments using targeted gene disruptions have shown that the loss of some transcription factors, such as PU.1, affects multiple hematopoietic lineages, whereas the loss of other transcriptional regulators specifically affects early B cell development (Scott et al., 1994). These transcription factors include EBF (early B cell factor), E2A, and BSAP (*Pax5*) (Bain et al., 1994; Urbanek et al., 1994; Zhuang et al., 1994; Lin and Grosschedl, 1995; Singh, 1996; Reya and Grosschedl, 1998).

Ebf encodes a B cell–specific transcription factor that binds DNA as a homodimer using a novel DNA-binding domain and a dimerization domain related to the helix-loop-helix (HLH) motif of the basic helix-loop-helix (bHLH) family of transcription factors (Hagman et al., 1993, 1995). Although the gene was named early B cell factor based on its cell type distribution, *Ebf* is also expressed in brain, adipose tissue, and olfactory epithelium, where it was independently identified (*Olf-1*) (Hagman et al., 1991, 1993; Wang and Reed, 1993). Targeted disruption of *Ebf* in mice results in animals with a severe defect in early B cell development, before any significant recombination of D_H-J_H segments has occurred in the immunoglobulin heavy chain locus (Lin and Grosschedl, 1995). Interestingly, *Ebf* heterozygous mice exhibit an approximately 2-fold decrease in the number of cells in the pro-B lymphocyte compartment, indicating that normal B cell development depends on the presence of two wild-type *Ebf* alleles.

E2A, the founding member of the bHLH family of transcription factors, is expressed ubiquitously in two splice forms, E12 and E47, which differ in their bHLH domains (Murre et al., 1989). E2A binds a DNA sequence termed E box (5′ CANNTG), which is found in the promoters of many different cell type-specific genes (Murre et al., 1994). Tissue specificity is, in part, determined by E2A dimerization partners, as described for the E47-MyoD interaction in myogenic differentiation (Murre et al., 1989; Lassar et al., 1991). A unique form of E2A exists specifically in B cells consisting of an E47 homodimer, termed BCF-1, or B cell factor-1 (Murre et al., 1991; Shen and Kadesch, 1995). Although E2A is widely expressed, targeted disruption of *E2a* produces viable animals that have a severe and specific defect in early B cell development, prior to the onset of Ig gene rearrangement (Bain et al., 1994; Zhuang et al., 1994). *E2a* heterozygous mutant mice also exhibit an approximately 2-fold decrease in the pro-B cell compartment.

The pronounced similarity of the B cell differentiation phenotype in *Ebf*$^{-/-}$ and *E2a*$^{-/-}$ mice led us to explore the possibility of cooperation between EBF and E2A in regulating a B cell–specific differentiation program. Ectopic expression of EBF and E2A in an immature hematopoietic cell line, Ba/F3, resulted in the transcriptional activation of the endogenous λ*5* and *VpreB* genes (Sigvardsson et al., 1997). The promoters of both the λ*5* and *VpreB* genes contain functional binding sites for EBF and E2A (Kudo et al., 1987; Sigvardsson et al., 1997). Strong transcriptional synergy was also observed in transient transfection assays, suggesting that EBF and E2A cooperate to directly activate at least two B cell–specific targets. Although these results suggested a collaborative role for these transcription factors in the regulation of B cell–specific genes in a tissue culture model, the experimental approach did not define a role for collaboration between EBF and E2A in normal B cell differentiation. Furthermore, we were unable to identify target genes for which EBF and E2A are necessary, but not sufficient for activation. The early arrest of B lymphocyte differentiation in the *Ebf* or *E2a* homozygous mutants also obscured the identification of additional target genes in vivo.

To examine the collaboration of EBF and E2A in vivo, we adopted a genetic approach analogous to synthetic

*To whom correspondence should be addressed (e-mail: rgross@lmb.uni-muenchen.de).
† Present address: Gene Center, Institute of Biochemistry, University of Munich, Feodor-Lynen Strasse 25, 81377 Munich, Germany.

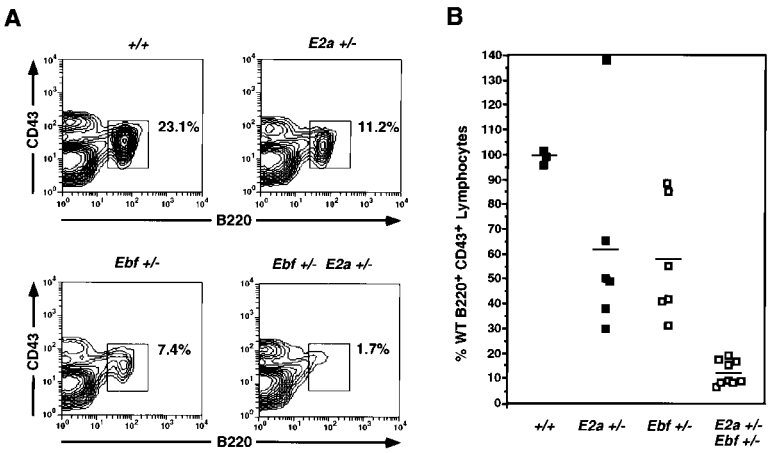

Figure 1. FACS Analysis of E17.5 Fetal Liver Pro-B Lymphocytes

(A) *Ebf*$^{+/-}$*E2a*$^{+/-}$ fetal livers contain fewer pro-B cells. Anti-CD43-FITC and anti-B220-PE antibodies were used to stain fetal liver cells from E17.5 littermates. Anti-B220 is used as a pan-B cell marker, while anti-CD43 stains only pro-B cells within the B cell population. Cells shown in the FACS plots have been gated on lymphocytic cells by forward and side scatter to exclude debris and large granular cells. Numbers next to each box represent the percentage of lymphocyte gated cells shown.

(B) Graphical representation of the percentage of mutant fetal liver pro-B cells, as compared to wild-type at E17.5 dpc. In each litter, the number of B220^{+} CD43^{+} cells in a wild-type animal was set at 100%; if there were two wild-type animals, an average of the two was used as 100%.

lethality experiments in yeast. In this approach, hypomorphic alleles of two distinct genes are combined and the offspring analyzed for a compound mutant phenotype. A synthetic effect of the two mutations would suggest that the gene products act in the same genetic pathway. Here, we show that *Ebf* and *E2a* act in the same genetic pathway by creating mice deficient in one allele of each gene. *Ebf/E2a* double heterozygous mice exhibit a marked defect in B cell differentiation. The analysis of these mice identifies new genetic targets of EBF and E2A and reveals a collaboration between these factors in normal B lymphopoiesis.

Results

Analysis of Ebf/E2a Double Heterozygous Mice Reveals a Defect in Early B Cell Development

Based on previous observations that pro-B cell differentiation is dependent on the gene dosage of *Ebf* or *E2a*, we predicted that we would be able to detect a synthetic effect in mice heterozygous for mutations in both *Ebf* and *E2a* genes (Zhuang et al., 1994; Lin and Grosschedl, 1995). *E2a*$^{+/-}$ and *Ebf*$^{+/-}$ mice were crossed to generate litters that contained wild-type, *Ebf*, and *E2a* single or double heterozygous pups. *Ebf*$^{+/-}$*E2a* pups exhibited reduced postnatal viability. Therefore, we examined B cell differentiation in fetal liver at E17.5 or E18.5, when double heterozygotes were represented in expected numbers.

Fetal liver cells were labeled with antibodies against the pan-B cell marker, B220, and CD43 (leukosialin), which is expressed on pro-B cells, and subjected to flow cytometry to determine the size of the pro-B cell compartment (Figure 1A). This analysis revealed a marked defect in the numbers of cells in the pro-B compartment of the *Ebf/E2a* double heterozygous embryos compared to wild-type embryos. *Ebf* or *E2a* single heterozygous pups exhibited a 2- to 3-fold decrease in pro-B cells relative to wild-type, consistent with previously published observations (Bain et al., 1994; Zhuang et al., 1994; Lin and Grosschedl, 1995). Analysis of multiple litters showed that *Ebf/E2a* double heterozygous fetal livers have, on average, a 9-fold reduction in the numbers of pro-B cells compared to wild-type littermates and a 4- to 5-fold reduction relative to single heterozygous littermate controls (Figure 1B). These data indicate that the transcription factors, EBF and E2A, cooperatively regulate early B cell development.

EBF/E2A Double Heterozygotes Exhibit a Defect in the Transition of Pro-B Cells from Fraction B to Fraction C

The extensive molecular and phenotypic characterization of B cell differentiation allowed us to further define the defect of *Ebf/E2a* double heterozygous mice (Li et al., 1993; Hardy and Hayakawa, 1995). Fetal liver cells were stained with antibodies to B220, CD43, HSA (heat stable antigen), and a fourth marker, either BP-1 or CD19, and analyzed by flow cytometry (Figure 2). The combination of these markers define pro-B cell populations as follows: the earliest population, Fraction A, is B220^{+}CD43^{+}HSA^{-}BP-1^{-}; Fraction B cells upregulate HSA to become B220^{+}CD43^{+}HSA^{+}BP-1^{-}; last, Fraction C cells upregulate BP-1 to become positive for all four surface antigens.

As anticipated, *Ebf* and *E2a* single heterozygous mice displayed an approximately 2-fold decrease specifically in the number of Fraction B pro-B cells (Figure 2A). This result is consistent with previous observations in homozygous single mutant mice that identified an absolute requirement for EBF or E2A in the transition from Fraction A to Fraction B (Bain et al., 1994; Zhuang et al., 1994; Lin and Grosschedl, 1995). The double heterozygous embryos showed a 4-fold decrease in the numbers of Fraction B cells, indicating an additive effect of the mutations in the *Ebf* and *E2a* genes. However, a significantly more pronounced decrease in the numbers of pro-B cells was observed in Fraction C of the *Ebf*$^{+/-}$*E2a*$^{+/-}$ mice in comparison to single heterozygotes (Figure 2A). From these data, we conclude that EBF and E2A not only act independently to regulate the transition from Fraction A to B as previously shown but act cooperatively to regulate further differentiation to Fraction C. Thus, these experiments reveal two distinct requirements for EBF and E2A in pro-B cell differentiation.

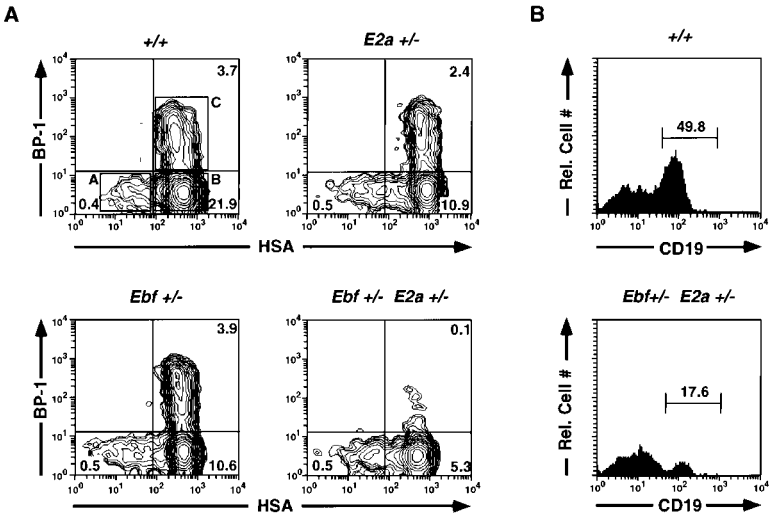

Figure 2. Four-Color FACS Analysis of Fetal Liver Pro-B Cell Differentiation

E18.5 fetal liver cells were stained with antibodies against HSA-FITC, CD43-PE, BP-1-biotin or CD19-biotin, and B220-APC to distinguish between pro-B cell fractions.

(A) *Ebf*$^{+/-}$*E2a*$^{+/-}$ fetal liver cells exhibit a defect in the Fraction B to C transition. B220^{+} CD43^{+} gated cells are shown. HSA^{-}BP-1^{-} cells represent Fraction A pro-B lymphocytes, HSA^{+}BP-1^{-} cells represent Fraction B, and HSA^{+}BP-1^{+} represent Fraction C. Numbers in each quadrant signify percentage of lymphocyte gated cells.

(B) *Ebf*$^{+/-}$*E2a*$^{+/-}$ fetal liver Fraction B cells express less CD19 on their surface than wild-type Fraction B cells. B220^{+} CD43^{+} BP-1^{-} gated cells are displayed in histograms. The number above the marker indicates percent of Fraction B cells that are CD19^{+}.

To further characterize changes within Fraction B that might indicate a problem in differentiation, we also analyzed the expression of CD19, considered a definitive marker of the B cell lineage (Rolink et al., 1996). This surface antigen is upregulated in Fraction B pro-B cells and is a target of the transcription factor, BSAP (*Pax5*) (Kozmik et al., 1992; Nutt et al., 1998). Compared to littermate controls, *Ebf/E2a* double heterozygous mice have fewer CD19^{+} cells and a lower percentage of CD19^{+} cells within Fraction B (Figure 2B). This decrease in the number of CD19-expressing cells may be due to decreased expression of the *CD19* gene itself or alternatively due to a defect in the differentiation of Fraction B cells. Whatever mechanism accounts for this defect, these data corroborate our conclusion that EBF and E2A are coordinately required for normal differentiation of Fraction B to Fraction C.

Adult Ebf$^{+/-}$E2a$^{+/-}$ Animals Show a Defect in Bone Marrow B Cell Development

Ebf/E2a double heterozygous mice that survived to adulthood were used to determine whether or not the fetal liver B cell defect was recapitulated in adult B lymphopoiesis. Analysis of bone marrow cells from 4-month-old mice with antibodies against B220 and IgM revealed that the B220^{lo}IgM^{-} population, which includes pro-B and pre-B cells, was significantly decreased in *Ebf*$^{+/-}$*E2a*$^{+/-}$ mice (Figure 3A). We note that bone marrow samples from *Ebf*$^{+/-}$ and *E2a*$^{+/-}$ single heterozygous mice contain more pro-B cells than the wild-type littermate, which is consistent with a delay at a subsequent stage in which cells are actively proliferating. The numbers of B220^{hi}IgM^{+} recirculating B lymphocytes are also reduced, although analysis of splenocytes clearly shows the presence of B cells (Figures 3A and 3B). In a separate experiment, analysis of the spleen revealed that B220^{+} lymphocytes in *Ebf*$^{+/-}$*E2a*$^{+/-}$ mice express both surface IgM and IgD (data not shown), suggesting that a certain percentage of developing B cells can progress to maturity and exit into the periphery. These data indicate that bone marrow B cell development is also affected in *Ebf/E2a* double heterozygous mice in a manner similar to that observed in the fetal liver.

Expression Levels of EBF and E47 but Not E12 Transcripts Are Constant in Pro-B Cells

One possible explanation for the differential defect between the first (Fraction A to B) and second (Fraction B to C) pro-B cell transitions would be a requirement for higher levels of EBF and E2A in Fraction B. To examine the expression levels of these genes during B cell differentiation, wild-type E17.5 fetal liver cells were labeled with antibodies against B220, CD43, HSA, and BP-1, and sorted by flow cytometry into three different pro-B cell Fractions, A, B, and C. The cDNA was normalized relative to the levels of actin transcripts and amplified in a linear range as shown for *IL7R* (Figure 4A). Levels of *Ebf* and *E47* transcripts remained constant in pro-B cell Fractions A, B, and C, while levels of *E12* transcripts increased from Fraction A to B (Figure 4B). If a requirement for higher levels of E12 caused the marked block in the transition of Fraction B to C in the *Ebf*$^{+/-}$*E2a*$^{+/-}$ fetal liver, we might also expect to detect a similar block of differentiation in the *E2a*$^{+/-}$ fetal liver as well, which we did not observe (Figure 2A). Therefore, the additional developmental defect in the Fraction B to C transition in *Ebf*$^{+/-}$*E2a*$^{+/-}$ mice is unlikely to be due to differential expression levels of either EBF or E2A. This developmental defect could, however, reflect a pronounced synergy in the function of EBF and E2A at this stage of differentiation or, alternatively, a collaboration of both transcription factors with another transcription factor.

Expression of Critical Genes in Fraction B Is Decreased in Ebf/E2a Double Heterozygotes

To understand the molecular basis of the B cell deficiency in *Ebf*/*E2a* double heterozygous mice, we examined the expression of multiple genes known or presumed to regulate B cell development. Toward this end, we performed RT-PCR analysis on B220^{+} cell populations that were sorted from the fetal liver of E15.5 embryos using magnetic beads (Figure 4D; Table 1). At this stage of mouse embryogenesis, very few pro-B cells have progressed to Fraction C, as defined by expression of the surface markers, BP-1 and HSA (Figure 4C). To control for a similar composition and number of sorted

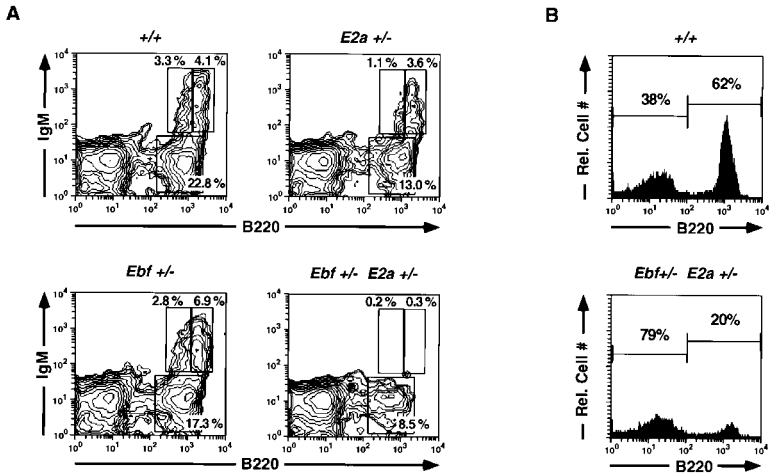

Figure 3. Adult *Ebf*$^{+/-}$*E2a*$^{+/-}$ Mice Display a Defect in B Cell Development

(A) Bone marrow from *Ebf*$^{+/-}$*E2a*$^{+/-}$ adult animals exhibits a pro-B cell defect. Bone marrow cells from 4-month-old littermates were stained with anti-B220-PE and anti-IgM-FITC. Early B cells are B220^{lo}IgM^{-}, immature B cells are B220^{lo}IgM^{+}, and mature recirculating cells B220^{hi}IgM^{+}. Numbers next to the black boxes indicate percentage of lymphocyte gated cells represented within the box.

(B) *Ebf*$^{+/-}$*E2a*$^{+/-}$ adult mice have fewer B lymphocytes in the spleen. Spleens from 4-month-old littermates were isolated and stained with anti-B220-PE antibodies. Numbers displayed above the markers represent percentage of lymphocyte gated cells.

B cells, we normalized the cDNA samples using RT-PCR to detect IL-7 receptor (*IL7R*) transcript levels, which were similar in FACS-sorted Fraction B cells of all genotypes (data not shown). Moreover, *IL7R* gene expression was previously shown to be unaffected in the *Ebf* homozygous mutant (Lin and Grosschedl, 1995). Aliquots of RT-PCR reactions were withdrawn at multiple cycles to ensure amplification in the linear range (data not shown).

Analysis of the previously identified targets for EBF and E2A, λ*5* and *VpreB*, revealed a significant reduction in *Ebf*$^{+/-}$*E2a*$^{+/-}$ fetal liver samples but not in wild-type, *Ebf*, or *E2a* single heterozygous controls (Figure 4D). We also examined expression of the *Pax5* gene, which has been shown to control the transition from Fraction B to C in adult bone marrow B cell development (Urbanek et al., 1994; Nutt et al., 1997). We found that in double heterozygous pro-B cells, *Pax5* expression was significantly decreased, suggesting that *Pax5* is genetically downstream of both *Ebf* and *E2a*. Other genes expressed at a reduced level in *Ebf*$^{+/-}$*E2a*$^{+/-}$ pro-B cells include the recombination activating genes, *Rag1* and *Rag2*. These data suggest that *Rag* gene expression, found in both B and T lymphocytes, is coordinately regulated by B cell–specific transcription factors, raising the possibility that these genes are regulated distinctly in

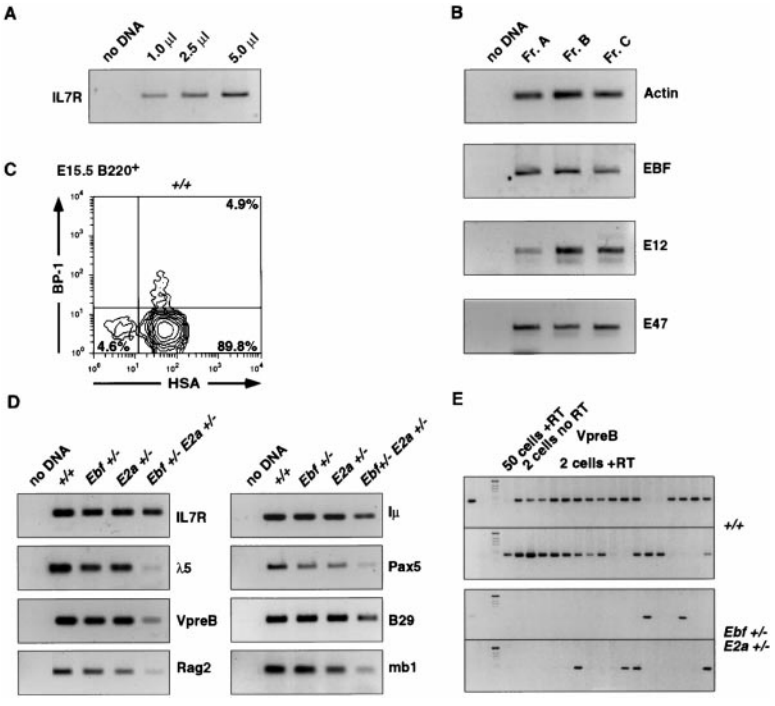

Figure 4. RT-PCR Analysis of Fetal Liver Pro-B Cells

(A) Semiquantitative bulk RT-PCR of wild-type E17.5 FACS sorted Fraction B pro-B cells shows that titration of input cDNA results in linear amplification of *IL7R* message after 30 cycles.

(B) RT-PCR analysis of transcript levels in wild-type E17.5 pro-B lymphocyte Fractions A, B, and C. Thirty cycle reactions are shown for EBF, E12, and E47; twenty cycles shown for actin.

(C) E15.5 fetal liver contains few Fraction C cells. FACS analysis of a representative +/+ fetal liver at E15.5, stained with anti-HSA-FITC, anti-B220-PE, and anti-BP-1-biotin. Only B220^{+} cells are shown; numbers in each quadrant indicate the percentage that each fraction represents of all B220^{+} cells.

(D) *Ebf*$^{+/-}$*E2a*$^{+/-}$ fetal liver cells exhibit a significantly altered profile of gene expression. Bulk RT-PCR analysis of fetal liver cells magnetically sorted with beads coated with anti-B220 antibody. Twenty-seven, thirty, and thirty-three cycles of PCR were performed for each set of primers to ensure amplification in the linear range. For *IL7R*, λ*5*, *VpreB*, *I*μ, *B29*, and *mb-1*, 30 cycles are shown; *Rag2* and *Pax5* PCR reactions shown are the product of 33 cycles.

(E) *Ebf*$^{+/-}$*E2a*$^{+/-}$ fetal livers have fewer VpreB-expressing cells. Fetal liver cells from E17.5 animals were stained with anti-B220-PE and anti-BP-1-biotin. B220^{+}BP-1^{-} cells were sorted into 96-well plates and subjected to RT-PCR analysis with primers to the VpreB gene. Each well represents two cells.

Table 1. Summary of RT-PCR Analysis

RT-PCR Summary			
IL7-R	++++	Bcl-2	++++
Iμ	++++	Bcl-xL	++++
Mu°	++++	PU.1	++++
λ5	+	BSAP	+
VpreB	++	CD19	++
SCID	++++	E12	+++
Rag1	+	LEF-1	++
Rag2	+	Sox-4	++++
mb-1	++	TCF-4	++++
B29	+++	Fgr	++++
Cdc25A	++++	Flk	++++
Cdc25B	++++	Blk	++++
Cdc25L	++++	Ikaros-VI	++++
	+/+	*Ebf*$^{+/-}$ E2a$^{+/-}$	Cell #
VpreB	28/36	6/36	2
Rag2	21/36	3/36	5

The symbols by each gene name represent the approximate level of expression seen in RT-PCR done on *Ebf*$^{+/-}$*E2a*$^{+/-}$ samples compared to a wild-type littermate control (++++). RT-PCR reactions that showed a difference in expression were repeated with the same samples to control for consistency, as well as with cDNA made from separate litters collected on different days. The last two rows of the table represent data from two- or five-cell RT-PCR analysis, an example of which is shown in Figure 4. The fractions represent the number of reactions giving a positive PCR result for VpreB or *Rag-2* out of total wells tested for that gene. All wells counted were positive in an *actin* RT-PCR assay.

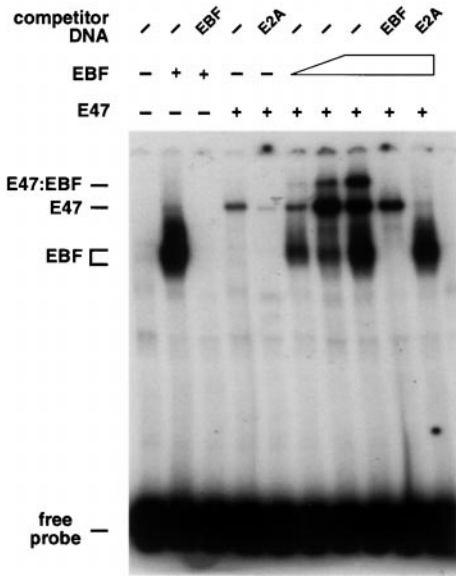

Figure 5. EBF and E47 Form a Ternary Complex on Sequences from the λ5 Promoter

An electrophoretic mobility shift assay using a 68 bp probe derived from the λ5 promoter, which contains one E box and one EBF site, is shown. In vitro transcribed and translated EBF and E47 proteins were added, along with 100-fold molar excess of competitor DNA where indicated. Competitor oligonucleotides contain either an EBF site from the *mb-1* promoter (EBF) or the μE5 E box from the Ig heavy chain enhancer (E2A). Complexes of different mobility are identified at the side of the panel.

both the lymphoid lineages. The introduction of a rearranged immunoglobulin transgene, encoding a HEL-specific heavy and light chain, was not able to rescue the defect in *Ebf*$^{+/-}$*E2a*$^{+/-}$ pro-B cell development (data not shown) as was observed for *Rag*$^{-/-}$ mice (Goodnow et al., 1988; Mason et al., 1992; Chen et al., 1994; Spanopoulou et al., 1994). *Mb-1* and *B29*, the signaling components of the surface immunoglobulin complex, were differentially affected by the loss of one *Ebf* and one *E2a* allele. *Mb-1* was expressed at a reduced level in *Ebf*$^{+/-}$*E2a*$^{+/-}$ pro-B cells, whereas *B29* transcription was only modestly affected. *B29* transcription has been observed in hematopoietic cell types other than B cells, whereas *mb-1* expression is confined to the B-cell lineage (Wang et al., 1998).

We also examined the expression of other candidate target genes known to be expressed in Fraction B cells that are involved in regulating gene expression, cell cycle progression, signaling, and cell survival (Table 1). Among these, only the transcription factor genes, *E12*, *Lef1*, and *Sox-4*, and the *CD19* gene were found to be modestly downregulated in *Ebf*$^{+/-}$*E2a*$^{+/-}$ pro-B cells. *Lef1* and *CD19* are downstream targets for BSAP and may be only indirectly regulated by EBF and E2A (Nutt et al., 1998). Taken together, these data identify a number of new genetic targets for the coordinate function of EBF and E2A in the development of the B cell lineage (summarized in Table 1).

The observed reductions in the RT-PCR assay on bulk sorted cell populations could reflect fewer pro-B cells expressing the target transcripts or a reduction in the level of target gene transcription in all of the *Ebf*$^{+/-}$*E2a*$^{+/-}$ pro-B cells. To distinguish between these two possibilities, we sorted several B220^{+}BP-1^{-} cells from either wild-type or *Ebf*$^{+/-}$*E2a*$^{+/-}$ fetal liver into each well of a 96-well plate and performed RT-PCR on these samples using primers for *VpreB* or *Rag-2* (Figure 4E; Table 1). In *Ebf*$^{+/-}$*E2a*$^{+/-}$ samples, we observed a 4- and 7-fold decrease, respectively, in the number of wells yielding a positive RT-PCR signal for *VpreB* or *Rag-2*, compared to wild-type. All wells shown were positive in an actin RT-PCR assay (data not shown). This analysis confirms the differences seen in the bulk RT-PCR assay and further suggests that a lower dose of both transcription factors reduces the frequency of gene activation in a cell population, rather than lowering the level of gene activation in all cells.

EBF and E47 Form a Ternary Complex on Sequences from the λ5 Enhancer/Promoter

The observation that EBF and E2A act coordinately in B cell development led us to examine whether this functional collaboration in vivo can be accounted for by cooperative DNA binding of these transcription factors. We had previously identified multiple EBF- and E2A-binding sites in the λ*5* enhancer/promoter, which were important for transactivation by EBF and E47 (Sigvardsson et al., 1997). This finding suggested that cooperation between EBF and E2A could, at least in part, be mediated directly at the promoter. To look for the formation of a ternary complex, we performed an electrophoretic mobility shift assay with sequences from the λ*5* promoter with in vitro translated EBF and E47 proteins (Figure 5). Both proteins were able to bind to the λ*5* sequence individually. Addition of increasing amounts of

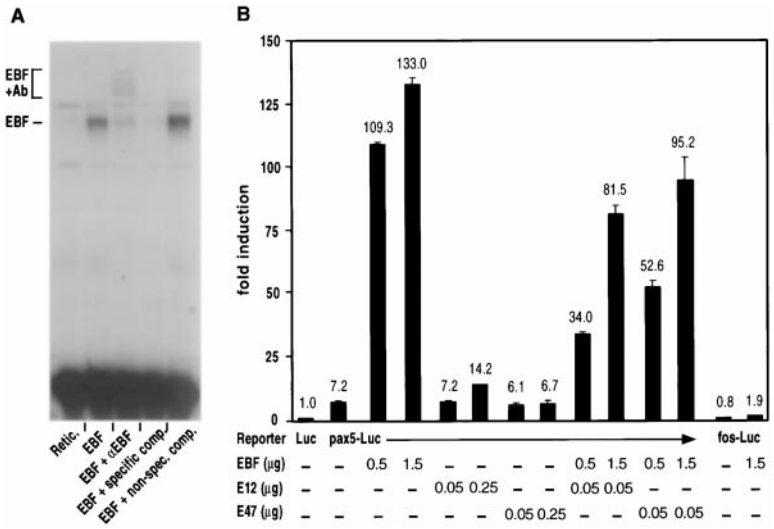

Figure 6. EBF Binds and Transactivates the *Pax5* Promoter

(A) An electrophoretic mobility shift assay using a 34 bp probe derived from the 1.8 kb *Pax5* promoter (GenBank #AF148961) is shown. In vitro transcribed and translated EBF was added, with a 100-fold molar excess of unlabeled competitor DNA where indicated. Unlabeled competitor oligonucleotides added were either the same DNA as used for the probe (*Pax5-EBF1*-specific) or DNA containing the μE5 E box (nonspecific). The supershifted EBF:DNA:Ab complex resulting from the addition of anti-EBF antiserum is indicated at the side of the panel.

(B) Transient transfections of NIH3T3 cells with EBF result in activation of the *Pax5* 1.8 kb promoter in a luciferase reporter construct. Fold induction represents relative light units (RLU) normalized by the expression of CMV-βgalactosidase and compared to the reporter vector alone. Each bar represents duplicate transfections, and the experiment shown is representative of three different experiments.

EBF added to E47 resulted in the formation of a slower migrating complex. This complex could be competed away by the addition of excess unlabeled oligonucleotides containing either an EBF- or E2A- binding site, suggesting that both EBF and E2A proteins were present in the complex. We therefore conclude that EBF and E47 are able to form a cocomplex using physiological binding sites, which may contribute to the cooperativity observed in vivo.

EBF Binds and Activates the B Cell–Specific Pax5 Promoter

Our observations of coordinate regulation of the λ*5* gene encouraged us to study a potentially more relevant target of EBF and E2A, the *Pax5* gene. The expression of *Pax5*, as well as the disruption of B cell development in *Pax5*$^{-/-}$ mice, are consistent with the *Pax5* gene being regulated by EBF and/or E2A (Adams et al., 1992; Urbanek et al., 1994). Furthermore, expression of E12 in a macrophage-like cell line results in the expression of both *Ebf* and *Pax5* transcripts (Kee and Murre, 1998). However, this study did not determine whether the *Pax5* gene is a direct target of either EBF or E2A. We therefore cloned 1.8 kb of sequence upstream of the murine B cell–specific start site in exon 1A of the *Pax5* gene (Busslinger et al., 1996). Analysis of the promoter identified multiple E box sequences, but none tested were found to bind to either in vitro translated E12 or E47. A putative EBF site (*Pax5-EBF1*) was identified 1121 bp upstream of the transcription start site and was used as a probe in an electrophoretic mobility shift assay (Figure 6A). In vitro translated EBF bound to the radioactively labeled *Pax5-EBF1* site and was supershifted by anti-EBF antisera. The EBF:DNA complex could be competed away by specific but not nonspecific unlabeled competitor oligonucleotides. These data suggest that EBF may regulate *Pax5* in vivo by binding directly to the promoter.

In order to test the function of the promoter, the 1.8 kb *Pax5* genomic fragment was cloned into a luciferase reporter construct. NIH3T3 cells were transiently transfected with the reporter construct together with plasmids expressing EBF or E2A (Figure 6B). EBF was able to transactivate the *Pax5* promoter up to 18-fold. E2A did not transactivate the *Pax5* promoter in this assay, consistent with a lack of functional E2A binding sites in the 1.8 kb fragment. Furthermore, E2A and EBF together did not activate to a greater degree than EBF alone. A covalently linked homodimer of E47 acted indistinguishably from E47 in this assay (data not shown). The decrease in activation seen in EBF/E2A combinations is most likely due to a nonspecific toxicity to the cells by the E2A proteins. All samples were normalized to the expression of CMV-βgalactosidase; however, we could not completely titrate out the observed nonspecific toxicity of the E2A proteins. Although we were unable to show direct regulation of the *Pax5* promoter by E2A, additional *cis*-regulatory elements may be required to allow the normal function of this promoter in B cells.

Discussion

Our experiments show that the loss of one allele each of *Ebf* and *E2a* markedly affects the differentiation of pro-B cells, compared to single heterozygous mice. Notably, the defect in double heterozygous mice occurs later in differentiation than that observed in single homozygous mutant mice. Moreover, transcription of multiple lymphocyte-specific genes is significantly affected in *Ebf*$^{+/-}$*E2a*$^{+/-}$ mice. We show that EBF and E2A can form a ternary complex at a target promoter, suggesting a possible contribution to functional cooperativity at the level of DNA binding. Finally, we show that EBF can directly bind and regulate the *Pax5* promoter. Together, these data suggest that coordinate regulation by distinct transcription factors is important in establishing temporal patterns of gene activation during B cell differentiation.

Cell type-specific gene expression can be achieved,

in principle, by transcriptional regulators that are dedicated to a given cell type or by the overlapping expression of transcription factors found in multiple cell types. Combinatorial gene control of differentiation has been well established for myogenesis in which two muscle-specific transcription factors, MEF2 and the bHLH protein, MyoD, cooperate to regulate specific gene expression (Molkentin et al., 1995). However, in B cell differentiation, no such collaboration between distinct transcription factors has been described to account for cell type-specific expression. The E2A gene products, E12 and E47, are expressed ubiquitously, although the homodimeric form of E47, termed BCF-1, is found only in B cells (Murre et al., 1991; Shen and Kadesch, 1995). Recent data suggest that formation of the E47 homodimers is regulated by the formation of intermolecular disulfide bonds and by protein dephosphorylation specifically in B cells (Benezra, 1994; Sloan et al., 1996). Conversely, the expression pattern of EBF is restricted to B cells and several other nonlymphoid cell types (Hagman et al., 1993; Wang and Reed, 1993; Garel et al., 1997). Ectopic expression of E47 alone in non-B cell lines does not result in the activation of B cell–specific genes, although E47 has been shown to weakly induce the expression of lymphoid genes such as *Rag1*, *Rag-2*, and *TdT* (Schlissel et al., 1991; Choi et al., 1996). Likewise, ectopic expression of EBF in non-B cells only weakly activates B cell–specific genes (Sigvardsson et al., 1997). In combination, however, EBF and E47 synergize to activate high levels of expression of two pro-B cell–specific genes, λ*5* and *VpreB* in non-B cell lines. As would be predicted, the λ*5* and *VpreB* genes were expressed in $Ebf^{+/-}E2a^{+/-}$ mice at very low levels, showing the synergy between EBF and E2A in an in vivo model.

Transcriptional synergy may be achieved by multiple mechanisms. First, DNA-binding proteins may bind cooperatively at a target promoter. We have been able to observe a cocomplex on λ*5* enhancer/promoter sequences, containing both EBF and E47. However, we have been unable to show any direct physical interaction between EBF and E2A in coimmunoprecipitation experiments using either in vitro translated proteins or B cell extracts (data not shown). It is possible that stable association of the two proteins may require an intermediary protein, as found in the association of the transcriptional coactivator, ALY with LEF-1 and AML-1, which regulate the TCRα enhancer (Bruhn et al., 1997).

Second, transcription factors can exhibit a functional synergy without physical interaction with each other, possibly by contacting components of the general transcription or chromatin remodeling machinery (Hernandez-Munain and Krangel, 1995). Consistent with this view, previous experiments using a cell line, stably transfected with EBF or E47, showed that EBF alone but not E47 could activate the endogenous λ*5* locus to a low level, although E47 has strong activating potential in transient transfection assays (Sigvardsson et al., 1997). In combination, however, both factors induced expression of the endogenous λ*5* locus to levels approaching those found in a pro-B cell line. These data suggest that the observed functional synergy may in part be due to the ability of EBF to bind its target in nuclear chromatin, perhaps making the locus available to the activating function of E2A. Finally, the λ*5* promoter sequence contains multiple EBF binding sites and E boxes that may be important for the synergistic function of these transcription factors. In the physiological process of B cell differentiation, functional cooperativity of EBF and E2A in activating target genes may be a combination of any of these mechanisms.

Finally, a possible mechanism for synergy might depend on the amount, or dose, of activators present. It had been previously observed that mice heterozygous for *Ebf* or *E2a* exhibit an approximately 2-fold decrease in the number of cells in the pro-B cell compartment, and the B cells were therefore sensitive to the gene dosage of *Ebf* or *E2a* (Zhuang et al., 1994; Lin and Grosschedl, 1995). Several examples of dose dependence have been described in mice in which a null mutation in one allele results in a mutant phenotype that is intermediate between the wild-type and homozygous mutant phenotypes, such as mice deficient in the gene encoding the transcriptional coactivator, p300 (Yao et al., 1998). At a molecular level, several mechanisms could account for the dose dependence of a gene encoding a transcription factor. The multiplicity of transcription factor binding sites in promoters, such as the λ*5* promoter, might impose dose dependence if occupancy of all binding sites is required for a transcriptional response and the factor binds in a noncooperative manner. Similarly, the heterozygosity of a gene encoding a rate-limiting factor in the assembly of a multi-protein complex would decrease formation of the functional complex (Henikoff, 1996). The appearance of a dose-dependent phenotype in mutant mice could also be the result of monoallelic expression of a critical transcription factor. Random inactivation of a critical gene in heterozygous mutant mice would confer a null phenotype on 50% of the cells. Recent data show that the *Pax5* gene is expressed in a monoallelic manner (Nutt et al., 1999). This is not the case for the *E2a* locus, since mature B cells from mice containing one *E2a*βgalactosidase knockin allele express both the wild-type E2A protein and the E2Aβgal fusion protein in the same cell (Zhuang et al., 1994). However, the possibility of monoallelic expression remains for the *Ebf* locus.

The specific stage at which pro-B cells from $Ebf^{+/-}E2a^{+/-}$ animals are deficient is consistent with a critical defect in transcription of either the *mb-1* or the *Pax5* genes, both of which affect the transition from Fraction B to Fraction C (Urbanek et al., 1994; Torres et al., 1996). This defect is earlier than the B cell developmental arrest observed in $Rag1^{-/-}$ and $Rag2^{-/-}$ mice (Mombaerts et al., 1992; Shinkai et al., 1992). Although *Rag1* and *Rag2* are also expressed in T lymphocytes, our observation that expression of the Rag genes is reduced in *Ebf/E2a* double heterozygous mice strongly suggests that these genes are distinctly regulated in B and T cells by lineage-specific factors, such as EBF or BCF-1. BSAP, a B lineage restricted transcription factor encoded by *Pax5*, is known to affect the expression of genes such as *CD19*, *mb-1*, *Lef1*, and *N-myc*, and binding sites for BSAP have been identified in the promoters of many other B cell–specific genes, including λ*5* and *VpreB* (Kozmik et al., 1992; Tian et al., 1997; Nutt et al., 1998). The requirement for BSAP in B cell differentiation in fetal liver is earlier than in adult bone marrow, since

no B220$^+$ cells are observed in the fetal liver of *Pax5* null mice (Nutt et al., 1997, 1998). However, recent data suggest that the activity of BSAP is concentration dependent, indicating that some functions of BSAP may be more susceptible to changes in BSAP levels than others (Wallin et al., 1998). In the functionally hypomorphic *Ebf/E2a* double heterozygotes, enough BSAP may be expressed to allow commitment to the B cell lineage but an insufficient amount to regulate the transition from Fraction B to C. Furthermore, both EBF and BSAP are regulators of the *mb-1* gene (Hagman et al., 1991; Fitzsimmons et al., 1996). Low levels of functional Igα, encoded by *mb-1*, may be insufficient to transduce important developmental signals. It has been reported that BSAP also regulates B cell proliferation (Wakatsuki et al., 1994). Likewise, EBF orthologs may be involved in cell cycle control, since the expression of *String*, a *cdc25*-like cell cycle regulator, in mitotic domain 2 coincides with the expression of *collier*, the *Drosophila* ortholog of EBF (Crozatier et al., 1996). However, we were unable to observe any change in the expression of the *CDC25* gene family members expressed in B lymphocytes (data not shown). Given the striking conservation between *collier* and *Ebf*, we would predict that some target genes would be conserved. It is intriguing to speculate that one conserved function of *Ebf* family members may be to regulate specific *Pax* genes. Our observations support a direct role for EBF in the activation of *Pax5*. Although we have not found any evidence for regulation of the *Pax5* promoter fragment by E2A, it is likely that another regulatory element, such as an enhancer, is also required for the proper expression of the *Pax5* gene that requires the activity of E2A and/or EBF. It has been reported that 12 kb of 5′ flanking sequence can direct high level B cell expression, although no specific regulatory regions have yet been characterized (Busslinger and Nutt, 1998). The finding that *Pax5* expression is coordinately regulated by EBF and E2A, as shown by our genetic experiments, suggests a hierarchy among transcription factors that are known to be involved in many different development processes (Murre et al., 1994; Mansouri et al., 1996; Wehr and Gruss, 1996).

The process of B cell differentiation appears to require EBF and E2A individually and coordinately at different stages of development. The transcriptional synergy of EBF and E2A in vivo may be important in establishing a specific temporal expression pattern within the B cell lineage. Temporal regulation of the *Pax5* gene by EBF and E2A may be of particular importance as recent studies have implicated *Pax5* in commitment to the B cell lineage (M. Busslinger, personal communication). Both EBF and E2A are expressed at the earliest stages of B cell differentiation and appear to individually regulate target genes at that stage. One of these target proteins may then facilitate the functional collaboration between EBF and E2A, which in concert would regulate target genes required at a later stage of differentiation. In particular, for the regulation of the transition of Fraction B to C of pro-B cell development, the synergistic action of EBF and E2A may also involve collaboration with *Pax5*. Such a mechanism that is dependent on the collaboration of multiple factors would increase the accuracy of temporal regulation of gene expression. Our in vivo cooperativity studies have not only identified some of the downstream genes for which both EBF and E2A are necessary but have also defined a novel physiological requirement for these two transcription factors in B cell development. Further studies will be required to pinpoint additional targets of EBF and E2A that promote progression through the early stages of B lymphocyte differentiation.

Experimental Procedures

Mice

E2aβgal$^{+/-}$ mice on a 129/Sv background were bred to *Ebf*$^{+/-}$ mice on a C57BL/6J background to generate litters for RNA and FACS analysis. Animals used for most experiments were bred in a nonbarrier facility. Mice were genotyped by PCR using the following conditions: *E2a* PCR, 94°C for 30 sec, 60°C for 30 sec, and 72°C for 1 min for 30 cycles; *Ebf* PCR, 94°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec for 30 cycles. The following primers were used for the genotyping PCR reactions: *E2a* PCR (1) 5′CCGAGCTCCTTAAAG GCCTCA, (2) 5′TTGTGGACATTTTCTAGGCAG, and (3) 5′GTTGTGC CCAGTCATAGCCG (Y. Zhuang); *Ebf* PCR (1) 5′GCCAACAGCGAAAA GACC, (2) 5′GGAGCCTCACCATTGCTGTAGAG, and (3) 5′ATGGCGA TGCCTGCTTGCCGAATA. Ig heavy and light chain (MD4) transgenic mice and primers were provided by J. Cyster. PCR primers for MD4 genotyping are as follows: IgH_{F1} 5′GCGACTCCATCACCAGCGAT; IgH_{F2} 5′CTGGAGCCCTAGCCAAGGAT; and IgH_{R1} 5′ACCACAGAC CAGCAGGCAGA.

Flow Cytometry

Fetal liver or bone marrow cells were collected in cold FACS buffer (1× PBS + 2% fetal calf serum) and homogenized with a syringe. Cells were layered over Lympholyte M (Cedarlane) and centrifuged to remove erythrocytes and cellular debris. Cells remaining at the interface were collected and washed with FACS buffer. For each antibody staining, 1×10^6 cells were placed in a well and incubated with anti-CD32/16 (anti-Fc receptor; PharMingen) for 10 min on ice to block nonspecific antibody staining. The cells were then washed and incubated with primary antibodies for 15 min on ice. For four-color analyses, cells were washed after primary staining and then incubated with streptavidin-Red613 (Gibco BRL) for 15 min on ice. The cells were washed one final time before resuspension in preparation for flow cytometric analysis. All analysis was done using Cellquest software (Becton-Dickinson). All of the antibodies used in flow cytometry experiments were obtained from PharMingen.

RT-PCR

Fetal liver cells were either magnetically sorted with beads coated with anti-mouse B220 antibody (Dynal) or FACS sorted (FACStar; Becton Dickinson). Each sample shown represents one fetal liver; no samples were pooled. For the two-cell RT-PCR experiment, cells were sorted into 96-well plates (MJ Research) containing RT lysis buffer as previously described (Klug et al., 1998). RNA for bulk RT-PCR was purified with Trizol (Gibco BRL). The samples were treated with RQ1 DNase (Promega) before inactivation by phenol/chloroform extraction and ethanol precipitation. The fetal liver bulk RNA samples were then reverse transcribed with MMLV-RT (Gibco BRL). The cDNA reactions were brought up to a final volume of 100 and 5 μl used in an RT-PCR reaction. All bulk RT-PCR reactions were standardized by levels of β-actin or by levels of IL-7 receptor. Standard RT-PCR conditions were as follows: 94°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec, using 0.1 μl of Taq polymerase (Boehringer Mannheim) per reaction for the number of cycles indicated in the figure legend. Aliquots were withdrawn at 27, 30, and 33 cycles to determine the minimum amplification necessary for detection. Primers used for bulk RT-PCR reactions have been described previously except for the following, which were designed using Geneworks software (Lin and Grosschedl, 1995; Wasserman et al., 1995): Cdc25A (GenBank accession number U27323) (1) 5′CAGAATCGAC CGAATCAGG, (2) 5′TGTCCAGAGGCTTACCATGC; Cdc25L (L16296) (1) 5′CCACTCTGTGACATGAACGC, (2) 5′CAGAGGAACAACAGGCT TCC; Cdc25B (D16237) (1) 5′CAAGAATGCTGTGAACCTGC, (2) 5′TG GTTCATGGGTCGGTAGTC; Tcf4 (IMAGE clones 444295/764951) (1)

5′CCAAGCAGGAAGCCTCCAGAGC, (2) 5′GAGGGACCATATGGGG AGGGAA; Sox4 (X70298) (1) 5′GGAGGCCCGGGATGCTCGCCCG ATG, (2) 5′GTCCGGGAATTCGAAGTGGGAGCCT; Lef1 (X58636) (1) 5′CTCCTGTAGCTTCTCTCTCTTCC, (2) 5′CAACACGAACAGAGAAA GGAGCA; BclXL (X83574) (1) 5′TGCGTGGAAAGCGTAGACAAGG, (2) 5′AGTGGATGGTCAGTGTCTGGTCAC; Ikaros VI (S74708) (1) 5′A CGAATGCTTGATGCCTCG, (2) 5′TTGTGAGGCTTACCAACGG; CD19 (M62542) (1) 5′AAGGAAGCGAATGACTGACC, (2) 5′TCTGAGCTCCA GTATCCTGG. Nested RT-PCR primers were used, except for actin, for the two-cell PCR reactions as follows: VpreB outer primers (X05556) (1) 5′TGGCCTATCTCACAGGTTGTG, (2) 5′ACGGCACAGT AATACACAGCC, VpreB inner primer (used with primer 1) (3) 5′GAAG TATCTCAGCAGGAACCTGG; Rag-2 outer primers (M64796) (1) 5′CA CATCCACAAGCAGGAAGTACAC, (2) 5′TCCCTCGACTATACACCA CGTCAA, Rag-2 inner primers (3) 5′CCTGCAGATGGTAACAGTGG, (4) 5′TCTTGCAACGACAGACATG; Actin (X03765) (1) 5′GACGACATG GAGAAGATCTGG, (2) 5′TGTGGTGGTGAAGCTGTAGC. VpreB primer 2, Rag-2 primer 2, and Actin primer 2 were included in the RT lysis buffer as RT primers. Three microliters of the 20 μl RT reaction was used in the first round of PCR with the outer primer set (primers 1 and 2) for 30 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec, using 0.1 μl of Taq polymerase per reaction. One μl of the first round reaction was used in the second round of PCR with the inner primer (VpreB primers 1 and 3; Rag-2 primers 3 and 4) set for 30 cycles, using the same conditions as the first. One round of 40 cycles using the actin PCR primers was performed using the conditions described above, as a sorting control. All PCR reactions were run on 2% agarose gels with ethidium bromide and visualized by UV transillumination. Images were captured with a gel documentation system (Alpha Innotech).

Electrophoretic Mobility Shift Assays

EBF and E47 cDNAs were transcribed and translated in vitro using the TNT Reticulocyte Lysate coupled transcription/translation system (Promega). The proteins were added together with unprogrammed reticulocyte lysate to make up a total of 4 μl of lysate within the gel shift reaction. 50,000 cpm ^{32}P-labeled probe was added to the samples in a cocktail as previously described, followed by competitor where appropriate (Hagman et al., 1991; Jacobs et al., 1994). The probe in Figure 5 is derived from sequences in the λ*5* promoter: 5′TCGAGTTCCATGGGGCAGGTGTTCAGTTGCTCACA GACCCAGGGGCCCTCAGGGACTGGATATCAGTCAGGC and 5′TC GAGCCTGACTGATATCCAGTCCCTGAGGGCCCCTGGGTCTGTGA GCAACTGAACACCTGCCCCATGGAAC (E47-3: −272 to −266; EBF-2: −213 to 197; some intervening sequence deleted (Sigvardsson et al., 1997). The probe used in Figure 6A, *Pax5-EBF1*, is contained in the *Pax5* promoter: 5′GGGGTGGGGGGACTCCCGGGAATCTACAG GCCAC and 5′GTGGCCTGTAGATTCCCGGGAGTCCCCCCACCCC. Gel shift conditions for Figure 6A were as previously described (Hagman et al., 1991; Jacobs et al., 1994). Double-stranded oligonucleotides containing either an *mb-1* site or a *μE5* site were used as competitor DNA, as previously described (Hagman et al., 1991; Jacobs et al., 1994). Reactions were incubated for 1 hr at room temperature and then run on a 5% nondenaturing acrylamide gel containing 5% glycerol.

Pax5 Promoter Cloning

1.8 kb of the *Pax5* promoter (Genbank #AF148961) was cloned from a mouse genomic library using the Mouse GenomeWalker Kit (Clontech). Two gene-specific primers containing sequence from *Pax5* exon 1A were used: (outer) 5′GGCCAGAACCAGGGAAAGGGTGTC AGC and (inner) 5′AAGGTGTGCAAGAGGCCCAGAGAGCAG.

Transient Transfections

DNA, including the construct containing the Pax5 promoter controlling the luciferase gene (derived from pGL3-B; Promega), was transiently transfected into NIH3T3 cells using Superfect (Qiagen). After 36 hr, cells were lysed and analyzed for βgalactosidase and luciferase activity using the substrate, chlorophenol red-β-D-galactopyranoside (Boehringer Mannheim), and a luciferase assay kit (Promega), respectively.

Acknowledgments

We are very grateful to Yuan Zhuang for the gift of the *E2aβgal* mice, as well as for many helpful comments and suggestions. We acknowledge Christian Ramers and Alia Ahmed for technical support, Paul Dazin, Bill Hyun, and Eric Wieder for help with FACS sorting, and Martha Gunthorpe for sequencing analysis. We thank Jason Cyster for providing the MD4 Ig transgenic mice, Art Weiss and Tannishtha Reya for critical reading of the manuscript, and members of the Grosschedl laboratory for helpful discussions. This work was supported by funds from the Howard Hughes Medical Institute.

Received October 12, 1998; revised June 1, 1999.

References

Adams, B., Dorfler, P., Aguzzi, A., Kozmik, Z., Urbanek, P., Maurer-Fogy, I., and Busslinger, M. (1992). Pax-5 encodes the transcription factor BSAP and is expressed in B lymphocytes, the developing CNS, and adult testis. Genes Dev. *6*, 1589–1607.

Bain, G., Maandag, E.C., Izon, D.J., Amsen, D., Kruisbeek, A.M., Weintraub, B.C., Krop, I., Schlissel, M.S., Feeney, A.J., van Roon, M., et al. (1994). E2A proteins are required for proper B cell development and initiation of immunoglobulin gene rearrangements. Cell *79*, 885–892.

Benezra, R. (1994). An intermolecular disulfide bond stabilizes E2A homodimers and is required for DNA binding at physiological temperatures. Cell *79*, 1057–1067.

Bruhn, L., Munnerlyn, A., and Grosschedl, R. (1997). ALY, a context-dependent coactivator of LEF-1 and AML-1, is required for TCRalpha enhancer function. Genes Dev. *11*, 640–653.

Busslinger, M., and Nutt, S. (1998). Role of the Transcription Factor BSAP (Pax-5) in B-cell Development. In Molecular Biology of B-cell and T-cell Development, J.G. Monroe and E.V. Rothenberg, eds. (Totowa, NJ: Humana Press), pp. 83–110.

Busslinger, M., Klix, N., Pfeffer, P., Graninger, P.G., and Kozmik, Z. (1996). Deregulation of PAX-5 by translocation of the Emu enhancer of the IgH locus adjacent to two alternative PAX-5 promoters in a diffuse large-cell lymphoma. Proc. Natl. Acad. Sci. USA *93*, 6129–6134.

Chen, J., Shinkai, Y., Young, F., and Alt, F.W. (1994). Probing immune functions in RAG-deficient mice. Curr. Opin. Immunol. *6*, 313–319.

Choi, J.K., Shen, C.P., Radomska, H.S., Eckhardt, L.A., and Kadesch, T. (1996). E47 activates the Ig-heavy chain and TdT loci in non-B cells. EMBO J. *15*, 5014–5021.

Crozatier, M., Valle, D., Dubois, L., Ibnsouda, S., and Vincent, A. (1996). Collier, a novel regulator of Drosophila head development, is expressed in a single mitotic domain. Curr. Biol. *6*, 707–718.

Fitzsimmons, D., Hodsdon, W., Wheat, W., Maira, S.M., Wasylyk, B., and Hagman, J. (1996). Pax-5 (BSAP) recruits Ets proto-oncogene family proteins to form functional ternary complexes on a B-cell-specific promoter. Genes Dev. *10*, 2198–2211.

Garel, S., Marin, F., Mattei, M.G., Vesque, C., Vincent, A., and Charnay, P. (1997). Family of Ebf/Olf-1-related genes potentially involved in neuronal differentiation and regional specification in the central nervous system. Dev. Dyn. *210*, 191–205.

Goodnow, C.C., Crosbie, J., Adelstein, S., Lavoie, T.B., Smith-Gill, S.J., Brink, R.A., Pritchard-Briscoe, H., Wotherspoon, J.S., Loblay, R.H., Raphael, K., et al. (1988). Altered immunoglobulin expression and functional silencing of self-reactive B lymphocytes in transgenic mice. Nature *334*, 676–682.

Hagman, J., Travis, A., and Grosschedl, R. (1991). A novel lineage-specific nuclear factor regulates mb-1 gene transcription at the early stages of B cell differentiation. EMBO J. *10*, 3409–3417.

Hagman, J., Belanger, C., Travis, A., Turck, C.W., and Grosschedl, R. (1993). Cloning and functional characterization of early B-cell factor, a regulator of lymphocyte-specific gene expression. Genes Dev. *7*, 760–773.

Hagman, J., Gutch, M.J., Lin, H., and Grosschedl, R. (1995). EBF contains a novel zinc coordination motif and multiple dimerization and transcriptional activation domains. EMBO J. *14*, 2907–2916.

Hardy, R.R., and Hayakawa, K. (1995). B-lineage differentiation stages resolved by multiparameter flow cytometry. Ann. NY Acad. Sci. *764*, 19–24.

Henikoff, S. (1996). Dosage-dependent modification of position-effect variegation in Drosophila. Bioessays *18*, 401–409.

Hernandez-Munain, C., and Krangel, M.S. (1995). c-Myb and core-binding factor/PEBP2 display functional synergy but bind independently to adjacent sites in the T-cell receptor delta enhancer [published erratum appears in Mol. Cell. Biol. 1995 Aug;15(8):4659]. Mol. Cell. Biol. *15*, 3090–3099.

Jacobs, Y., Xin, X.Q., Dorshkind, K., and Nelson, C. (1994). Pan/E2A expression precedes immunoglobulin heavy-chain expression during B lymphopoiesis in nontransformed cells, and Pan/E2A proteins are not detected in myeloid cells. Mol. Cell. Biol. *14*, 4087–4096.

Kee, B.L., and Murre, C. (1998). Induction of early B cell factor (EBF) and multiple B lineage genes by the basic helix-loop-helix transcription factor E12. J. Exp. Med. *188*, 699–713.

Klug, C.A., Morrison, S.J., Masek, M., Hahm, K., Smale, S.T., and Weissman, I.L. (1998). Hematopoietic stem cells and lymphoid progenitors express different Ikaros isoforms, and Ikaros is localized to heterochromatin in immature lymphocytes. Proc. Natl. Acad. Sci. USA *95*, 657–662.

Kozmik, Z., Wang, S., Dorfler, P., Adams, B., and Busslinger, M. (1992). The promoter of the CD19 gene is a target for the B-cell-specific transcription factor BSAP. Mol. Cell. Biol. *12*, 2662–2672.

Kudo, A., Sakaguchi, N., and Melchers, F. (1987). Organization of the murine Ig-related lambda 5 gene transcribed selectively in pre-B lymphocytes [published erratum appears in EMBO J. 1987 Dec 20; 6(13):4242]. EMBO J. *6*, 103–107.

Lassar, A.B., Davis, R.L., Wright, W.E., Kadesch, T., Murre, C., Voronova, A., Baltimore, D., and Weintraub, H. (1991). Functional activity of myogenic HLH proteins requires hetero-oligomerization with E12/E47-like proteins in vivo. Cell *66*, 305–315.

Li, Y.S., Hayakawa, K., and Hardy, R.R. (1993). The regulated expression of B lineage associated genes during B cell differentiation in bone marrow and fetal liver. J. Exp. Med. *178*, 951–960.

Lin, H., and Grosschedl, R. (1995). Failure of B-cell differentiation in mice lacking the transcription factor EBF. Nature *376*, 263–267.

Mansouri, A., Hallonet, M., and Gruss, P. (1996). Pax genes and their roles in cell differentiation and development. Curr. Opin. Cell. Biol. *8*, 851–857.

Mason, D.Y., Jones, M., and Goodnow, C.C. (1992). Development and follicular localization of tolerant B lymphocytes in lysozyme/anti-lysozyme IgM/IgD transgenic mice. Int. Immunol. *4*, 163–175.

Molkentin, J.D., Black, B.L., Martin, J.F., and Olson, E.N. (1995). Cooperative activation of muscle gene expression by MEF2 and myogenic bHLH proteins. Cell *83*, 1125–1136.

Mombaerts, P., Iacomini, J., Johnson, R.S., Herrup, K., Tonegawa, S., and Papaioannou, V.E. (1992). RAG-1-deficient mice have no mature B and T lymphocytes. Cell *68*, 869–877.

Murre, C., McCaw, P.S., Vaessin, H., Caudy, M., Jan, L.Y., Jan, Y.N., Cabrera, C.V., Buskin, J.N., Hauschka, S.D., Lassar, A.B., et al. (1989). Interactions between heterologous helix-loop-helix proteins generate complexes that bind specifically to a common DNA sequence. Cell *58*, 537–544.

Murre, C., McCaw, P.S., and Baltimore, D. (1989). A new DNA binding and dimerization motif in immunoglobulin enhancer binding, daughterless, MyoD, and myc proteins. Cell *56*, 777–783.

Murre, C., Voronova, A., and Baltimore, D. (1991). B-cell- and myocyte-specific E2-box-binding factors contain E12/E47-like subunits. Mol. Cell. Biol. *11*, 1156–1160.

Murre, C., Bain, G., van Dijk, M.A., Engel, I., Furnari, B.A., Massari, M.E., Matthews, J.R., Quong, M.W., Rivera, R.R., and Stuiver, M.H. (1994). Structure and function of helix-loop-helix proteins. Biochim. Biophys. Acta *1218*, 129–135.

Nutt, S.L., Urbanek, P., Rolink, A., and Busslinger, M. (1997). Essential functions of Pax5 (BSAP) in pro-B cell development: difference between fetal and adult B lymphopoiesis and reduced V-to-DJ recombination at the IgH locus. Genes Dev. *11*, 476–491.

Nutt, S.L., Morrison, A.M., Dorfler, P., Rolink, A., and Busslinger, M. (1998). Identification of BSAP (Pax-5) target genes in early B-cell development by loss- and gain-of-function experiments. EMBO J. *17*, 2319–2333.

Nutt, S.L., Vambrie, S., Steinlein, P., Kozmik, Z., Rolink, A., Weith, A., and Busslinger, M. (1999). Independent regulation of the two Pax5 alleles during B-cell development. Nat. Genet. *21*, 390–395.

Reya, T., and Grosschedl, R. (1998). Transcriptional regulation of B-cell differentiation. Curr. Opin. Immunol. *10*, 158–165.

Rolink, A., and Melchers, F. (1993). B lymphopoiesis in the mouse. Adv. Immunol. *53*, 123–156.

Rolink, A., ten Boekel, E., Melchers, F., Fearon, D.T., Krop, I., and Andersson, J. (1996). A subpopulation of B220+ cells in murine bone marrow does not express CD19 and contains natural killer cell progenitors. J. Exp. Med. *183*, 187–194.

Schlissel, M., Voronova, A., and Baltimore, D. (1991). Helix-loop-helix transcription factor E47 activates germ-line immunoglobulin heavy-chain gene transcription and rearrangement in a pre-T-cell line. Genes Dev. *5*, 1367–1376.

Scott, E.W., Simon, M.C., Anastasi, J., and Singh, H. (1994). Requirement of transcription factor PU.1 in the development of multiple hematopoietic lineages. Science *265*, 1573–1577.

Shen, C.P., and Kadesch, T. (1995). B-cell-specific DNA binding by an E47 homodimer. Mol. Cell. Biol. *15*, 4518–4524.

Shinkai, Y., Rathbun, G., Lam, K.P., Oltz, E.M., Stewart, V., Mendelsohn, M., Charron, J., Datta, M., Young, F., Stall, A.M., et al. (1992). RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. Cell *68*, 855–867.

Sigvardsson, M., O'Riordan, M., and Grosschedl, R. (1997). EBF and E47 collaborate to induce expression of the endogenous immunoglobulin surrogate light chain genes. Immunity *7*, 25–36.

Singh, H. (1996). Gene targeting reveals a hierarchy of transcription factors regulating specification of lymphoid cell fates. Curr. Opin. Immunol. *8*, 160–165.

Sloan, S.R., Shen, C.P., McCarrick-Walmsley, R., and Kadesch, T. (1996). Phosphorylation of E47 as a potential determinant of B-cell-specific activity. Mol. Cell. Biol. *16*, 6900–6908.

Spanopoulou, E., Roman, C.A., Corcoran, L.M., Schlissel, M.S., Silver, D.P., Nemazee, D., Nussenzweig, M.C., Shinton, S.A., Hardy, R.R., and Baltimore, D. (1994). Functional immunoglobulin transgenes guide ordered B-cell differentiation in Rag-1-deficient mice. Genes Dev. *8*, 1030–1042.

Tian, J., Okabe, T., Miyazaki, T., Takeshita, S., and Kudo, A. (1997). Pax-5 is identical to EBB-1/KLP and binds to the VpreB and lambda5 promoters as well as the KI and KII sites upstream of the Jkappa genes. Eur. J. Immunol. *27*, 750–755.

Torres, R.M., Flaswinkel, H., Reth, M., and Rajewsky, K. (1996). Aberrant B cell development and immune response in mice with a compromised BCR complex. Science *272*, 1804–1808.

Urbanek, P., Wang, Z.Q., Fetka, I., Wagner, E.F., and Busslinger, M. (1994). Complete block of early B cell differentiation and altered patterning of the posterior midbrain in mice lacking Pax5/BSAP. Cell *79*, 901–912.

Wakatsuki, Y., Neurath, M.F., Max, E.E., and Strober, W. (1994). The B cell-specific transcription factor BSAP regulates B cell proliferation. J. Exp. Med. *179*, 1099–1108.

Wallin, J.J., Gackstetter, E.R., and Koshland, M.E. (1998). Dependence of BSAP repressor and activator functions on BSAP concentration. Science *279*, 1961–1964.

Wang, M.M., and Reed, R.R. (1993). Molecular cloning of the olfactory neuronal transcription factor Olf-1 by genetic selection in yeast. Nature *364*, 121–126.

Wang, H., Diamond, R.A., and Rothenberg, E.V. (1998). Cross-lineage expression of Ig-beta (B29) in thymocytes: positive and negative gene regulation to establish T cell identity. Proc. Natl. Acad. Sci. USA *95*, 6831–6836.

Wasserman, R., Li, Y.S., and Hardy, R.R. (1995). Differential expression of the blk and ret tyrosine kinases during B lineage development is dependent on Ig rearrangement. J. Immunol. *155*, 644–651.

Wehr, R., and Gruss, P. (1996). Pax and vertebrate development. Int. J. Dev. Biol. *40*, 369–377.

Yao, T.P., Oh, S.P., Fuchs, M., Zhou, N.D., Ch'ng, L.E., Newsome, D., Bronson, R.T., Li, E., Livingston, D.M., and Eckner, R. (1998). Gene dosage-dependent embryonic development and proliferation defects in mice lacking the transcriptional integrator p300. Cell *93*, 361–372.

Zhuang, Y., Soriano, P., and Weintraub, H. (1994). The helix-loop-helix gene E2A is required for B cell formation. Cell *79*, 875–884.

GenBank Accession Number

The GenBank accession number for the sequence reported in this paper is AF148961.