Early B Cell Factor Promotes B Lymphopoiesis with Reduced Interleukin 7 Responsiveness in the Absence of E2A

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Abstract

The basic helix-loop-helix transcription factors encoded by the E2A gene function at the apex of a transcriptional hierarchy involving E2A, early B cell factor (EBF), and Pax5, which is essential for B lymphopoiesis. In committed B lineage progenitors, E2A proteins have also been shown to regulate many lineage-associated genes. Herein, we demonstrate that the block in B lymphopoiesis imposed by the absence of E2A can be overcome by expression of EBF, but not Pax5, indicating that EBF is the essential target of E2A required for development of B lineage progenitors. Our data demonstrate that EBF, in synergy with low levels of alternative E2A–related proteins (E proteins), is sufficient to promote expression of most B lineage genes. Remarkably, however, we find that E2A proteins are required for interleukin 7–dependent proliferation due, in part, to a role for E2A in optimal expression of N-*myc*. Therefore, high levels of E protein activity are essential for the activation of EBF and N-*myc*, whereas lower levels of E protein activity, in synergy with other B lineage transcription factors, are sufficient for expression of most B lineage genes.

Key words: lymphopoiesis • transcription factor • E2A • EBF

Introduction

The development of B lymphocytes from multipotent hematopoietic progenitors depends on appropriate expression of lineage-associated genes, rearrangement of IgH and light chain gene segments, and responsiveness to stromal cell–derived cytokines (1). B lineage specification and commitment is orchestrated by multiple transcriptional regulatory proteins that function in a complex network to control gene expression (2, 3). The basic helix-loop-helix (bHLH) transcription factors encoded by the E2A gene are absolutely required for B lymphocyte development from the earliest identifiable progenitors (4, 5). There are four E proteins in mammals, E12 and E47, which are alternatively spliced products of the E2A gene, HEB, and E2-2, all of which bind the E-box motif (CANNTG; 6). These four proteins share a high degree of sequence identity in the bHLH domain, which is required for DNA binding and protein dimerization, and in the two known activation domains. In B lymphocyte progenitors (BLPs), the major E-box binding protein is E47, consistent with the specific requirement for E2A proteins in B lymphocyte development (7, 8).

Three independent strains of E2A-deficient mice have been created and all have an identical phenotype: a complete absence of B220^{+} CD19^{+} cells (Fraction B), Ig gene rearrangements, and most B lineage–associated gene expression in the BM and fetal liver (FL; 4, 5, 9). Mice lacking HEB or E2-2 do not have an overt B lymphocyte defect, however, mice that are heterozygous for mutations in E2A and HEB or E2-2 have a notable reduction in B lymphopoiesis compared with E2A-heterozygous mice (10). It is currently not known whether the distinct E proteins are functionally redundant, although this hypothesis is supported by the observation that B lymphocytes develop in E2A-deficient mice that express HEB from the E2A genomic locus (11).

The E proteins were cloned based on their ability to bind to sequences in the IgH and Igκ enhancers (12, 13). In fact, E2A proteins are sufficient to induce the expression of IgH and Igκ loci in many cell types (14–16). In addition, they have been implicated in expression of the RAGs (RAG-1 and RAG-2), terminal deoxynucleotide trans-

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ferase, and early B cell factor (EBF), a transcription factor required for B lymphopoiesis at the same stage of development as E2A (17, 18). E2A and EBF cooperatively regulate the expression of the surrogate light chain proteins λ5 and VpreB and the signal-transducing protein mb-1 (Ig-α; 19, 20). The transcription factor Pax5, which is essential for B lineage commitment, but not B lineage specification, has been proposed to be a target of EBF and possibly E2A (21). These observations have led to the conclusion that a transcriptional hierarchy exists in which E2A proteins induce the expression of EBF followed by Pax5 and consequently B lineage specification and commitment (2, 3).

In this study we have tested the hypothesis that EBF is the essential target of E2A required for the development of BLPs by expressing EBF in hematopoietic progenitors from E2A-deficient mice. If EBF is the only essential target of E2A at this stage of development, we would predict that expression of EBF would be sufficient to promote B lineage specification and commitment in the absence of E2A. Alternatively, E2A may have many essential targets in BLPs in which case EBF may fail to rescue, or only rescue some aspects, of BLP development. Moreover, a partial rescue could provide important insights into the functions of E2A in B lymphopoiesis. Our experiments indicate that induction of EBF is the major function of E2A in promoting the specification of BLPs. However, E2A proteins are also required to establish an appropriate cellular context for optimal induction of N-*myc* and responsiveness to the lymphopoietic cytokine IL-7. In addition, we find that distinct thresholds of E protein activity are required for the induction of EBF and N-*myc* as compared with other B lineage genes. Taken together, our data demonstrate that E2A proteins provide a central link between the induction of B lineage–specific gene expression and expansion of BLPs.

Materials and Methods

Mice and Genotyping. Mice were housed at the University of Chicago Animal Resource Center. E47^{−/−} mice have been described previously (9). The E47 mutation was identified by PCR using the primers E47bas forward 5′-CAGATGAGGTGCT-GTCCCGGA-3′ and E47bas reverse 5′-CAGGATCACCTG-CACCGCCT-3′ as described previously (9).

Isolation, Infection, and Culture of FL Progenitors. FLs were harvested from E47^{−/−} and E47^{+/−} embryos between E12 and E13 after the mating of E47^{−/−} male mice with E47^{+/−} female mice. Pooled FL populations were depleted of cells expressing Gr-1, CD11b, and Ter119 (Lin[−]) by incubation with biotinylated antibodies (BD Biosciences) followed by streptavidin magnetic beads. They were then passed over a magnetic column (Miltenyi Biotec). In the case of FL cells used for ex vivo examination of EBF and Pax5, mRNA levels the Lin[−] cocktail also included CD19. Depleted FL cells were spin infected for 2 h at 10^6 cells/ml of viral supernatant containing the S003 (green fluorescent protein [GFP]), S003EBF (EBF), or MigR1-Pax5 (Pax5) retrovirus as described previously (22). The cells were then cultured on a subconfluent layer of irradiated S17 stromal cells (2,000 rads) in OPTI-MEM supplemented with 10% FBS, 2×10^{-5} M 2-mercaptoethanol, $1\times$ penicillin/streptomycin/glutamine, a 1:100 di-

lution of IL-7 supernatant (provided by F. Melchers, University of Basel, Basel, Switzerland), and a 1:500 dilution of c-*kit* ligand (KL; Genetics Institute). Retroviral supernatants were produced from Pheonix cells as described previously (23).

Subsequent infection of E47^{+/−};GFP or E47^{−/−};EBF cells with S003ERId3, S003ERId3m, pCS-ret (a derivative of S003 in which GFP was replaced with the extracellular domain of human CD25), or PCS-Id3 were performed as described above. FL progenitors used for infection with the S003ERId3 or S003ERId3m retroviruses were isolated from C57Bl/6 embryos at E13 and cultured under the conditions described above for 15 d before infection. 36 h after infection, GFP^{+} cells were isolated on a cell sorter and cultured for an additional 20 h before the addition of 1 μM 17β-estradiol for 6 h. Limiting dilution assays were performed as described previously (24, 25).

Construction of Retroviral Vectors. S003EBF was created by ligation of rat EBF (provided by R. Reed, Johns Hopkins School of Medicine, Baltimore, MD) into the S003 retroviral vector using XhoI and NotI. S003ERId3 and S003ERId3m were cloned by PCR amplification of Id3 or Id3m from TAT-Id3 or TAT-Id3m using PCR primers 5′HA(EcoRI); 5′-GGAATTCACCATGT-CCGGCTATCCATATGACGTCCCAGACTATGCTGGCT-CCATGGCCGGTACC-3′ and TATI-d3rev (22). The amplified fragment was blunt end ligated into the EcoRV site of pBSK and then isolated by digestion with EcoRI. The EcoRI fragment was cloned into pBSKER digested with EcoRI, thereby ligating HA-Id3 or HA-Id3m in-frame with the estrogen hormone binding domain. The ERId3 and ERId3m fragments were isolated from pBSKERId3 and pBSKERId3m by digestion with XhoI and NotI, and ligated to S003 digested with XhoI and NotI producing S003ERId3 and S003ERId3m. pCS-Id3 was created by ligation of Id3 digested with EcoRI into the EcoRI site of pCS-ret.

Flow Cytometry. Cells were stained with anti–B220-cy5, anti–CD19-PE, anti–BP-1-PE (BD Biosciences) as described previously (26). Staining with anti–bromodeoxyuridine (BrdU)-FITC (eBioscience) and propidium iodide (PI) for cell cycle analysis was performed as described by Kee et al. (22) after the addition of 1 μM BrdU to cells in culture for 20 min. The cells were analyzed on a FACSCalibur™ (Becton Dickinson) using the CELLQuest™ Pro software package.

Real Time RT-PCR Analysis. Total RNA was isolated using Trizol (Invitrogen) primed with oligo dT$_{18}$ and was reverse transcribed with M-MuLV reverse transcriptase (New England Biolabs, Inc.). Intron-spanning PCR primers were designed to avoid amplification of genomic DNA, whereas product sizes were kept to ∼100 bp to maximize amplification efficiency. Each PCR reaction was set up with first strand cDNA, HPLC-purified primers, passive reference dye, and SYBR Green QPCR Master Mix (Stratagene) according to the manufacturer's instructions. Real time PCR was performed in replicates of three to five on an MX-4000 instrument (Stratagene) at 95°C for 10 min, followed by 40 cycles at 95°C for 30 s, 58°C for 1 min, and 72°C for 1 min. Flurometric data were collected at the end of the annealing step in each cycle, and a dissociation curve was performed at the end of 40 cycles to confirm specificity of amplification. Data were recorded using software supplied by the manufacturer and analyzed using Microsoft Excel. cDNA from the pro–B cell line 38B9 was used at known relative amounts to construct a seven-point standard curve for each primer pair in each run. Standard curves for all primer pairs showed a linear decrease in threshold cycle proportional to the log of relative cDNA input, with correlation coefficients ranging from 0.9904 to 0.9995. Relative starting cDNA amounts for unknowns were calculated from the corresponding

standard curve and expressed in arbitrary units relative to expression of the endogenous reference gene HPRT. The primers used include: E12 sense 5′-GTGGCCGTCATCCTCAGC, E12 anti-sense 5′-GCTGCTTTGGGGTTCAGG; E47 sense 5′-GCCG-AAGAGGACAAGAAGG, E47 anti-sense 5′-CTTCTCCTC-CAGGGACAGC; HEB sense 5′-CGATTAGGAACCCACGA-AGG, HEB anti-sense 5′-GGAAAATGAGCCTCTCTCTGAT; and HPRT sense 5′-ACCTCTCGAAGTGTTGGATA, HPRT anti-sense 5′-CAACAACAAACTTGTCTGGA. RT-PCR was performed as described previously (9). Control experiments were performed with RNA isolated from cells lines with known expression levels of each of the tested genes to confirm the specificity of the real time PCR reactions.

Northern Blot Analysis. Isolation of RNA and Northern blot analysis were performed as described in Kee et al. (22) using cloned PCR–amplified cDNA fragments from the N-*myc*, c-*myc*, IL-7Rα, and actin genes.

Electromobility Shift Analysis (EMSA). Whole cell extracts were prepared by freezing cell pellets at −70°C for 2–24 h followed by lysis in Solution C (20 mM Hepes, pH 7.4, 200 mM NaCl, 1 mM EDTA, 1 mM EGTA) containing 1% NP-40 and phosphatase and protease inhibitor cocktails (Sigma-Aldrich). The lysates were vortexed vigorously for 2 min and incubated for an additional 15 min with rotation at 4°C. Double stranded DNA probes were labeled and EMSA was performed exactly as described in Kee et al. (17). The sequences of the μE5 (13), EBF

(27), Pax5 (28), and Oct (29) oligonucleotides have been published previously.

Results

E47$^{-/-}$ FL Cells Fail to Develop into BLPs and Do Not Express EBF. We examined FL cells from E47$^{+/-}$ and E47$^{-/-}$ embryos at E15.5 for expression of B220, CD19, and c-*kit*, markers of early B lineage progenitors. E47$^{-/-}$ mice are E2A deficient because cells from these mice do not express detectable levels of E12 or E47 protein (9). Consistent with previous, studies we found that B220$^+$ CD19$^+$ and B220$^+$ c-*kit*$^+$ cells are absent from E47$^{-/-}$ FL at E15.5, whereas these cells are present in E47$^{+/-}$ FL (Fig. 1 A). We hypothesized that the absence of B lineage cells in E47$^{-/-}$ FL might be due to a requirement for E2A to promote EBF expression. In support of this hypothesis, we found that E47$^{-/-}$ Lin$^-$ FL cells isolated at E14.5 lack EBF mRNA, whereas EBF was detected in E47$^{+/+}$ or E47$^{+/-}$ Lin$^-$ FL (Fig. 1 B). Similarly, we found that Pax5 mRNA was not detected in E14.5 E47$^{-/-}$ Lin$^-$ FL, consistent with the notion that EBF is required for Pax5 expression.

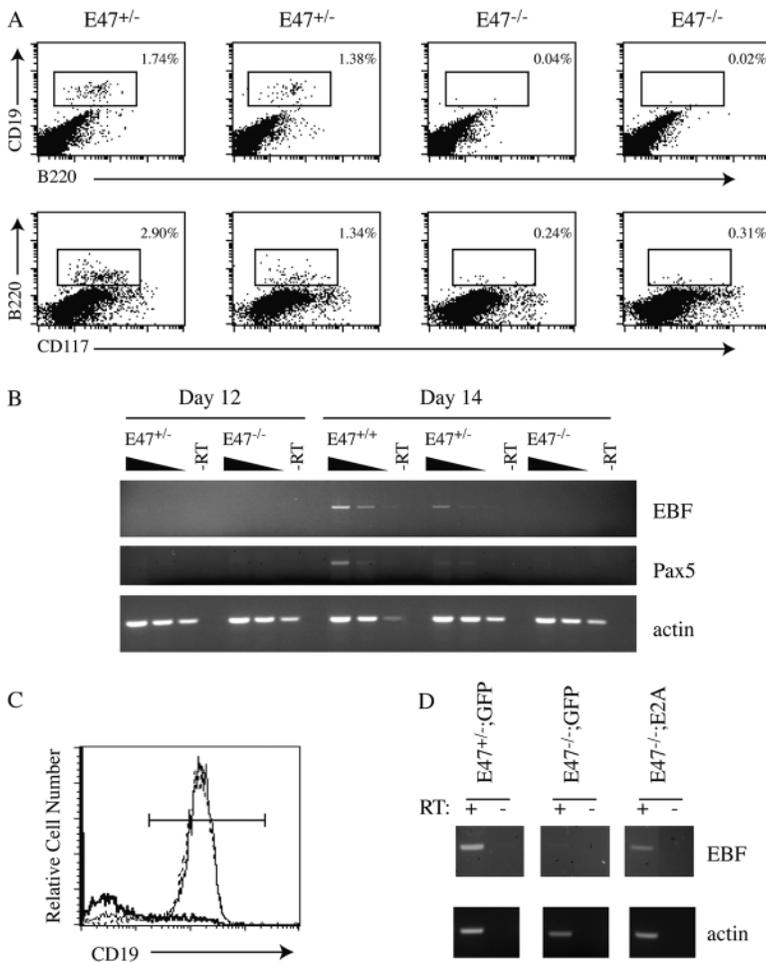To study B lymphopoiesis from E47$^{-/-}$ progenitors, we used an in vitro culture system that supports development

Figure 1. E2A proteins are required for development of BLPs and expression of EBF and Pax5 in FL. (A) FACS® analysis for B220 and CD19 (top) or c-*kit* (bottom)– expressing cells in the liver E47$^{+/-}$ and E47$^{-/-}$ embryos at E15.5. (B) Semiquantitative RT-PCR analysis of EBF, Pax5, and actin mRNA in Lin$^-$ cells isolated from E47$^{+/+}$, E47$^{+/-}$, or E47$^{-/-}$ FL at E12.5 or E14.5. Threefold serial dilutions of cDNA, starting at 50 ng/reaction were amplified for 32 cycles (EBF), 35 cycles (Pax5), or 28 cycles (actin). In all experiments, RNA that had not been reverse transcribed (−RT) served as a negative control. (C) FACS® analysis of CD19 expression on E47$^{-/-}$;GFP (dark line), E47$^{+/-}$;GFP (dotted line), and E47$^{-/-}$;E2A (light line) FL cells 18 d after infection. (D) RT-PCR analysis of EBF and actin mRNA from E47$^{+/-}$;GFP, E47$^{-/-}$;GFP, and E47$^{-/-}$;E2A cells 15 d after infection. 15 ng cDNA was amplified for 30 cycles (EBF) or 28 cycles (actin). In all RT-PCR experiments, the lanes being compared were amplified in parallel and run on the same gel, although the lanes have been rearranged to aid in comparison.

of BLPs from multipotent hematopoietic progenitors (24). Lin$^-$ FL progenitors were isolated from embryos at E12.5 and cultured on a subconfluent layer of irradiated S17 stromal cells in media containing KL and IL-7. At E12.5, EBF and Pax5 expression are not yet readily detected in Lin$^-$ cells (Fig. 1 B). Under these conditions, E47$^{+/-}$ progenitors give rise to a detectable number of CD19$^+$ lymphoid cells within 7–9 d of culture (Fig. 1 C). By contrast, E47$^{-/-}$ FL cells fail to give rise to a CD19$^+$ lymphoid population (Fig. 1 C). In fact, E47$^{-/-}$ FL progenitors underwent only a minor expansion over the first 10–15 d of culture. After this time, the cells became adherent and resembled macrophages, with the majority expressing CD11b. However, coinfection of E47$^{-/-}$ Lin$^-$ FL cells with retroviruses producing E12 and E47 was sufficient to rescue the development of CD19$^+$ cells (Fig. 1 C). E47$^{+/-}$ FL cells infected with a control retrovirus producing GFP only (E47$^{+/-}$; GFP) and E47$^{-/-}$ FL cells expressing E2A (E47$^{-/-}$;E2A) both expressed EBF mRNA by RT-PCR (Fig. 1 D). Analysis of EBF expression in E47$^{-/-}$;GFP cultures was complicated by the fact that the stromal cells on which these progenitors are growing express high levels of EBF. Because E47$^{-/-}$;GFP cells fail to undergo extensive expansion, the recovered cells are highly contaminated with stromal cells. Regardless, EBF expression was significantly lower (approximately sixfold) in the E47$^{-/-}$;GFP cultures

(Fig. 1 D). Therefore, expression of E2A, but not GFP, in E47$^{-/-}$ Lin$^-$ FL progenitors is sufficient to induce the expression of EBF.

EBF, But Not Pax5, Rescues the Development of B220$^+$ CD19$^+$ Cells from E47$^{-/-}$ Progenitors. We tested the hypothesis that EBF is the essential target of E2A leading to B lymphocyte development by introducing EBF into E47$^{-/-}$ Lin$^-$ FL progenitors and assessing the ability of these cells to give rise to BLPs in vitro. Remarkably, expression of EBF was sufficient to promote the development of B220$^+$ CD19$^+$ cells from E47$^{-/-}$ progenitors (Fig. 2 A). In addition, E47$^{-/-}$;EBF cells underwent significant expansion resulting in an ~80-fold increase in cell numbers by day 18 of culture. By contrast, nonadherent cells in E47$^{-/-}$;GFP cultures increased only fourfold during this time (Fig. 2 B). By day 20 of culture, the majority of cells in E47$^{+/-}$;GFP and E47$^{-/-}$;EBF cultures expressed CD19 and B220 and >70% also expressed the BLP-associated aminopeptidase BP-1/6C3 (Fig. 2 C). No CD19$^+$ lymphoid cells were detected in cultures of E47$^{-/-}$ progenitors even after 40 d of continuous culture on S17 + KL + IL-7, demonstrating that there are no rare progenitors present in E47$^{-/-}$ FL capable of B lineage differentiation under these conditions (unpublished data). Therefore, EBF is sufficient to promote the development of CD19$^+$ lymphoid cells from E47$^{-/-}$ FL progenitors.
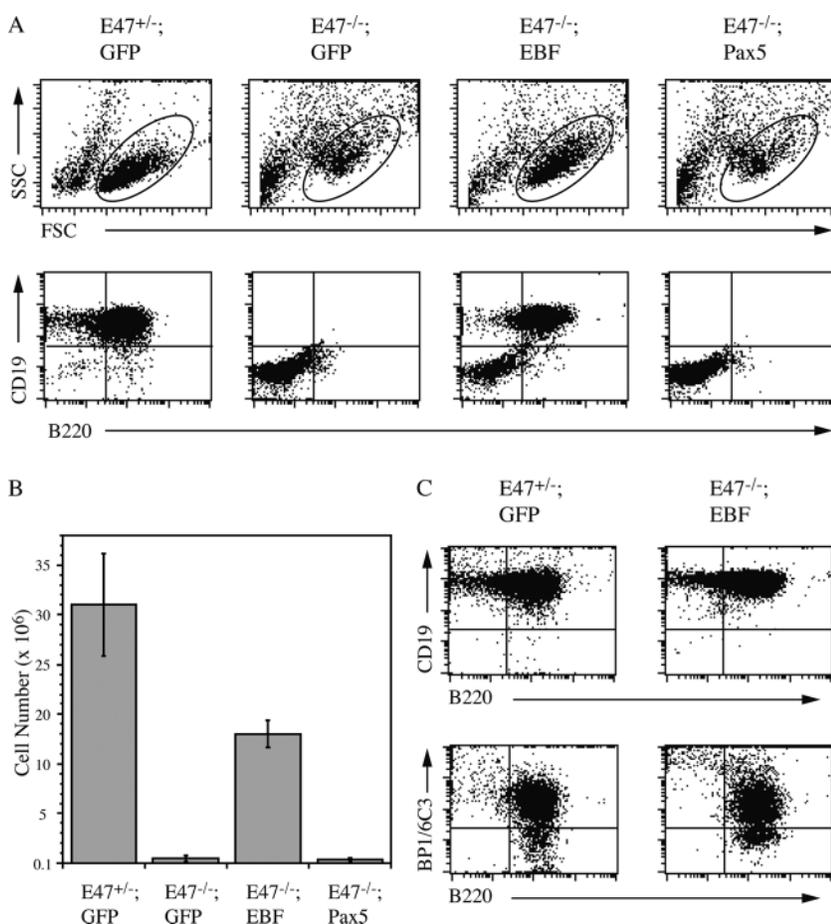

Figure 2. Development of B220$^+$ CD19$^+$ BP1$^+$ cells from E47$^{-/-}$ FL progenitors expressing EBF, but not Pax5. (A) Hematopoietic progenitors were isolated from E47$^{+/-}$ and E47$^{-/-}$ FL cells at day 12 of gestation, infected with the GFP only, EBF, or Pax5-producing retrovirus, and cultured on S17 stromal cells in the presence of KL and IL-7. 12 d after the initiation of culture, the resulting cell populations were isolated and stained with antibodies recognizing CD11b, Gr-1, B220, and CD19. The plots show B220 and CD19 expression on CD11b$^-$ Gr-1$^-$ cells. (B) 18 d after the initiation of culture, the total number of cells that had developed from the starting population was determined. (C) 20 d after the initiation of culture, the indicated cell populations were stained with antibodies recognizing B220, CD19, or BP-1/6C3.

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We also asked whether Pax5, which functions downstream of E2A and EBF in the transcriptional hierarchy leading to B lineage commitment, could rescue the development of $CD19^+$ cells from $E47^{-/-}$ progenitors. Remarkably, infection of $E47^{-/-}$ progenitors with a retrovirus producing Pax5 did not lead to significant expansion of these cells or expression of CD19 (Fig. 2, A and B).

Expression of B Lineage Genes in $E47^{-/-}$;EBF BLPs. To determine whether $E47^{-/-}$;EBF or $E47^{-/-}$;Pax5 cells expressed B lineage genes we analyzed total RNA isolated from these cells by RT-PCR. Surprisingly, most B lineage genes could be detected in RNA isolated from $E47^{-/-}$;EBF cells including those previously shown to be dependent on E2A such as $\lambda5$ and mb-1 (Fig. 3 A). By contrast, RT-PCR of RNA isolated from $E47^{-/-}$;GFP and $E47^{-/-}$;Pax5 cells did not reveal expression of any B lineage genes (Fig. 3 A). To compare the level of B lineage gene expression in $E47^{+/-}$;GFP and $E47^{-/-}$;EBF cells directly, we isolated RNA from sorted $CD19^+$ cells and performed a semiquantitative RT-PCR (Fig. 3 B). Slight differences in the level of mRNA were observed for some of these genes between the two cell types, particularly for the RAG-2 gene. However these differences are within the range of error of our RT-PCR reactions (two- to threefold). Therefore, expression of EBF appears to be sufficient to promote the expression of most B lineage genes in the absence of E2A.

IgH Chain Rearrangement in $E47^{-/-}$;EBF BLPs. Next, we examined the state of rearrangement of the IgH locus, another hallmark of B lineage differentiation that is thought to be E2A dependent. First, we examined DNA from $E47^{+/-}$; GFP and $E47^{-/-}$;EBF BLPs for the presence of germline IgH

alleles using PCR primers specific for J_{H4} and intronic DNA 5′ of the J_H gene segments (Mu0), which is deleted during D to J_H rearrangement (30). We found a similar level of germline IgH loci in the two populations of cells (Fig. 3 C). Subsequently, we examined rearrangements of D and J_H gene segments using degenerate primers (D_HL and D_HR) that detect 9 of the 10 D gene segments and a J_H4 primer (30). Using this assay, rearrangement of D gene segments to all four J_H gene segments could be detected in DNA from $E47^{+/-}$;GFP BLPs (Fig. 3 D). Remarkably, a similar pattern of D-J_H rearrangements was detected in DNA from $E47^{-/-}$;EBF BLPs (Fig. 3 D). The level of rearrangement, as judged by the intensity of each rearranged fragment, also appeared to be similar between the two populations. Therefore, $E47^{-/-}$;EBF BLPs are able to rearrange their IgH D and J_H gene segments.

We also examined rearrangements of the V to DJ_H gene segments using degenerate PCR primers specific for three families of V genes: V_{J558}, V_{7183}, and V_{Q52} (30). Rearrangements to all three V gene families were observed in DNA isolated from $E47^{+/-}$;GFP and $E47^{-/-}$;EBF BLPs (Fig. 3 E). Therefore, $E47^{-/-}$;EBF BLPs are capable of V-DJ_H rearrangement.

EBF Does Not Induce Expression of HEB or E2-2. We questioned whether EBF might promote B lymphocyte development by inducing the expression of alternative E-box binding proteins. To test this possibility, we measured HEB and E2-2 mRNA levels in sorted $B220^+$ $CD19^+$ $E47^{+/-}$;GFP and $E47^{-/-}$;EBF cells by real time RT-PCR. Both HEB and E2-2 mRNA were increased only 1.3-fold in $E47^{-/-}$;EBF compared with $E47^{+/-}$;GFP cells, indicating that EBF is not leading to a dramatic increase in HEB
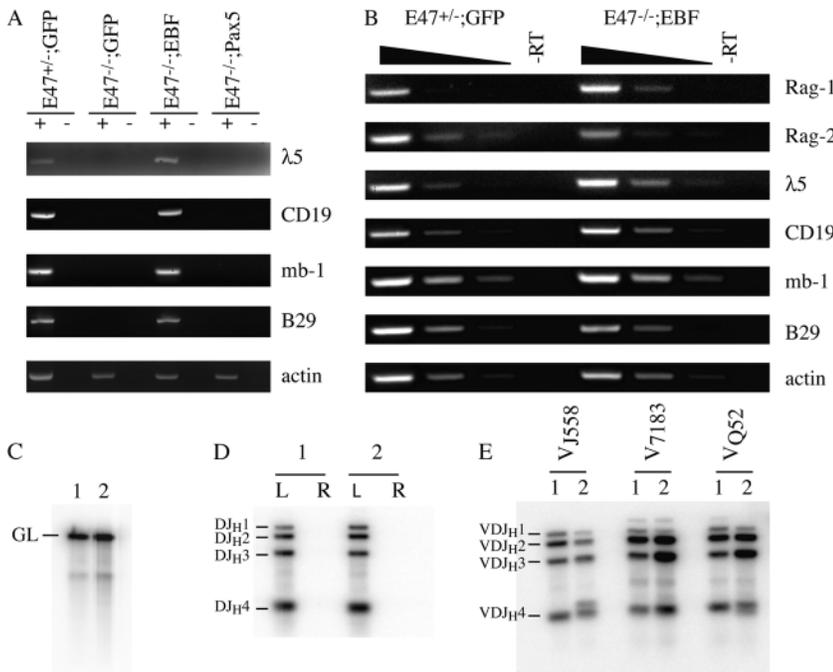
Figure 3. Expression of B lineage genes and IgH rearrangements in $E47^{-/-}$;EBF BLPs. (A) RT-PCR analysis of B lineage gene expression in RNA isolated from $E47^{+/-}$;GFP, $E47^{-/-}$;GFP, $E47^{-/-}$; EBF, and $E47^{-/-}$;Pax5 populations 20 d after infection. The input cDNA was standardized such that a similar amount of actin was amplified under semiquantitative conditions. In all RT-PCR experiments, the lanes being compared were amplified in parallel and run on the same gel. The +RT and −RT lanes were juxtaposed to aid in comparison. (B) $B220^+$ $CD19^+$ cells were sorted from cultures of $E47^{+/-}$;GFP and $E47^{-/-}$;EBF cells and total RNA was extracted. Semiquantitative RT-PCR was performed on serial threefold dilutions of reverse transcribed RNA, 50 ng/reaction, using the indicated PCR primers. In all experiments, RNA that had not been reverse transcribed (−RT) served as a negative control. (C) PCR-amplified products from DNA isolated from $B220^+$ $CD19^+$ $E47^{+/-}$; GFP (lane 1) and $E47^{-/-}$;EBF (lane 2) BLPs using primers to detect the germline (GL) configuration of the IgH locus, (D) the J_H4 primer in conjunction with the D_HL (L) primer, which detects D-J_H rearrangements, or the D_HR (R) primer, which is in the opposite orientation and should not amplify D-JH rearrangements, and (E) the V_{J558}, V_{7183}, and V_{Q52} primers in combination with the J_H4 primer. All amplified products were transferred to nylon membrane and probed with a radiolabeled cDNA fragment containing a DJ_H4 fragment.
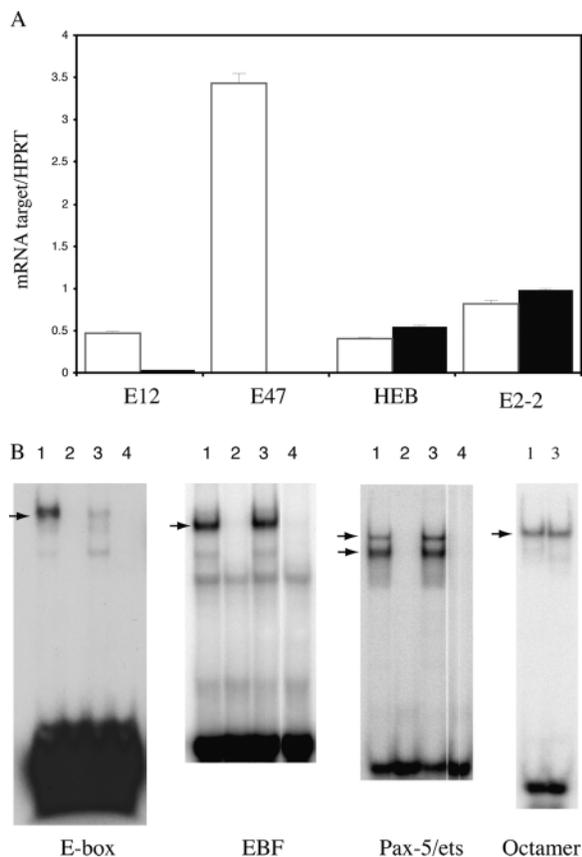
A
E47+/-;GFP E47-/-;GFP E47-/-;EBF E47-/-;Pax5
+ −   + −   + −   + −
λ5
CD19
mb-1
B29
actin

B
E47+/-;GFP   E47-/-;EBF
−RT   −RT
Rag-1
Rag-2
λ5
CD19
mb-1
B29
actin

C   D   1   2   E   VJ558   V7183   VQ52
1 2   L R L R   1 2   1 2   1 2
GL   DJH1   VDJH1
          DJH2   VDJH2
          DJH3   VDJH3
          DJH4   VDJH4

Figure 4. EBF does not induce E protein mRNA or protein in E47$^{-/-}$;EBF cells. (A) Total RNA isolated from sorted B220$^+$ CD19$^+$ E47$^{+/-}$;GFP (open bars) and E47$^{-/-}$;EBF (solid bars) cells at day 20 of culture was analyzed by real time PCR for expression of E12, E47, HEB, and E2-2. The abundance of target mRNA relative to the level of HPRT mRNA is shown. Error bars represent the standard error of five replicate samples. (B) EMSA of whole cell protein extracts prepared from sorted B220$^+$ CD19$^+$ E47$^{+/-}$;GFP (lanes 1 and 2) and E47$^{-/-}$;EBF (lanes 3 and 4) cells 25 d after the initiation of culture. The extracts were incubated with the probe in the presence of a noncompetitive (lanes 1 and 3) or competitive (lanes 2 and 4) oligonucleotide, except in the case of the Oct probe, where no competitive oligonucleotide was added. Lanes 1–4 for each of the probes were run on the same gel. The probes used include the E-box–containing μE5 probe, the EBF site from the mb-1 promoter, a composite Pax5/ets site from the mb-1 promoter, and an Octamer protein binding sequence as indicated at the bottom of the panel.

or E2-2 expression (Fig. 4 A). We also found that EBF did not restore expression of E12 because E12 mRNA remained 16-fold lower in E47$^{-/-}$;EBF than in E47$^{+/-}$;GFP BLPs (Fig. 4 A). E47 mRNA was not detected in the E47$^{-/-}$;EBF cells because these cells contain a targeted deletion of the exon encoding the E47 bHLH domain (Fig. 4 A). Therefore, EBF does not promote the development of B220$^+$ CD19$^+$ cells from E47$^{-/-}$ progenitors by inducing the expression of alternative E protein genes.

Expression of EBF and Pax5 with Reduced E-box Binding Proteins in E47$^{-/-}$;EBF BLPs. We examined whole cell extracts from these sorted populations by EMSA and found a dramatic reduction (approximately eightfold) in E-box binding proteins in E47$^{-/-}$;EBF cells compared with the

E47$^{+/-}$;GFP cells (Fig. 4 B). We also examined these extracts for EBF and Pax5 protein. Remarkably, both EBF and Pax5 were present in E47$^{-/-}$;EBF extracts at levels comparable to the E47$^{+/-}$;GFP extracts (Fig. 4 B). Octamer binding proteins were also present at equivalent levels in both populations, consistent with the presence of these proteins in all cell types (Fig. 4 B). Therefore, ectopic expression of EBF in E47$^{-/-}$ hematopoietic progenitors is sufficient to promote the development of B220$^+$ CD19$^+$ cells that have low levels of E-box binding proteins and levels of EBF and Pax5 that are comparable to E47$^{+/-}$ BLPs. Moreover, the expression of Pax5 in the E47$^{-/-}$; EBF cells indicates that these cells are committed to the B lymphocyte lineage (31, 32). Consistent with this hypothesis we have not been able to generate macrophages from CD19$^+$ E47$^{-/-}$;EBF cells in cultures containing S17 + KL + M–CSF (unpublished data).

E47$^{-/-}$;EBF BLPs Require E Protein Activity for Survival and Expression of B Lineage Genes. The expression of B lineage genes in E47$^{-/-}$;EBF BLPs was surprising because many of these genes have been shown previously to be targets of E2A. In addition, E protein activity is required for the survival of BLPs (22). Therefore, our data indicated that either: (a) expression of EBF is able to overcome the need for E proteins in BLPs or (b) that alternative E proteins are expressed at levels sufficient to compensate for the loss of E2A. The level of expression of HEB and E2-2, and E-box binding activity, is low in E47$^{-/-}$;EBF BLPs. However, it is possible that they are present at sufficient levels to promote expression of many genes. To determine whether E protein activity is required for survival or B lineage gene expression in E47$^{-/-}$;EBF BLPs, we infected these cells with a retrovirus that produces an estradiol-inducible E protein antagonist Id3 (ERId3). As a control, the cells were infected with a retrovirus producing a mutant form of Id3 (ERId3m). Remarkably, E47$^{-/-}$;EBF cells were more sensitive to E protein inhibition than E47$^{+/-}$;GFP cells as demonstrated by the increased proportion of cells staining with annexin V 9 h after induction of ERId3 (Fig. 5 A). In a similar experiment, E47$^{+/-}$;GFP and E47$^{-/-}$;EBF BLPs were infected with an Id3-producing virus (in this case coexpressing the extracellular domain of human CD25 instead of GFP) or hCD25-producing virus and hCD25$^+$ cells were isolated by flow cytometry 20 h after infection. RT-PCR analysis of RNA isolated from these cells revealed that Id3 caused a decrease in expression of RAG-1 and RAG-2, but not CD19, mRNA in E47$^{-/-}$;EBF cells (Fig. 5 B). Therefore, the low level of HEB and E2-2 present in E47$^{-/-}$;EBF cells is required for the survival of BLPs and expression of the RAG genes.

E47$^{-/-}$;EBF BLPs Have Reduced Responsiveness to IL-7. Our data demonstrate that EBF is the major target of E2A required for B lineage specification. However, one apparent difference between cultures of E47$^{+/-}$;GFP and E47$^{-/-}$;EBF BLPs was the rate at which cell numbers increased over time. Although a significant number of E47$^{-/-}$;EBF BLPs were generated by days 12–15 of culture, the subsequent expansion
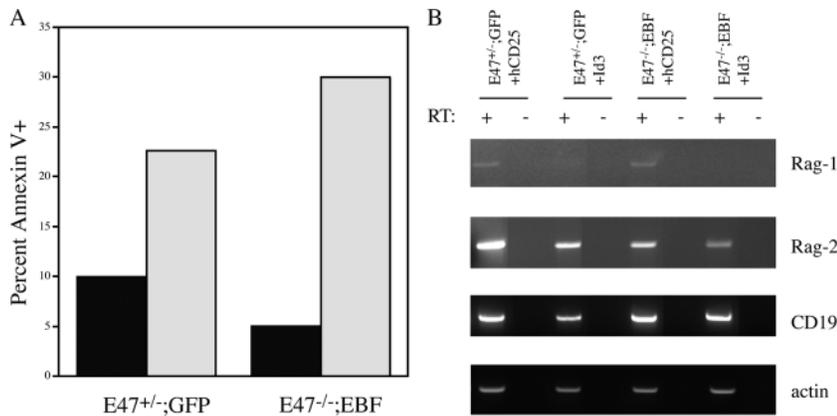
Figure 5. E47$^{-/-}$;EBF BLPs require E protein activity for survival and expression of RAG-1 and RAG-2. (A) E47$^{+/-}$;GFP and E47$^{-/-}$;EBF BLPs were infected with ERId3m (solid bars) or ERId3 (gray bars). 24 h after infection, the cells were treated with 1 μM estradiol for 9 h and the percent of GFP^{hi} cells binding annexin V was determined by flow cytometry. (B) E47$^{+/-}$;GFP and E47$^{-/-}$; EBF BLPs were infected with hCD25- or Id3/hCD25-producing retrovirus. 24 h after infection, hCD25$^+$ cells were isolated and total RNA was extracted and reverse transcribed for analysis by semi-quantitative RT-PCR. Expression of RAG-1, RAG-2, CD19, and actin are shown. In all RT-PCR experiments, the lanes being compared were amplified in parallel and run on the same gel. The +RT and −RT lanes were juxtaposed after photographing the gel.

of these cells was much slower than E47$^{+/-}$;GFP BLPs. To determine why there were fewer cells in cultures of E47$^{-/-}$; EBF BLPs, we examined their cloning efficiency compared with E47$^{+/-}$;GFP cells under limiting dilution conditions in the presence of S17 + KL + IL-7 or IL-7 alone. In three independent experiments, we found that the frequency of purified B220$^+$ CD19$^+$ cells that could proliferate in response to IL-7 in the presence of S17 + KL was similar (within twofold) between E47$^{+/-}$;GFP and E47$^{-/-}$;EBF CD19$^+$ cells, although the colony size was consistently smaller in the latter cultures (Table I). Remarkably, the frequency of E47$^{-/-}$; EBF BLPs that could respond to IL-7 alone was dramatically lower (between 5- and 10-fold) than E47$^{+/-}$;GFP cells with only a few rare progenitors proliferating enough to score as a positive response (Table I). After 4 d, no viable cells remained in E47$^{-/-}$;EBF IL-7, only cultures. Therefore, E47$^{-/-}$;EBF BLPs do not respond appropriately to IL-7.

To examine further the ability of E47$^{-/-}$;EBF BLPs to respond to IL-7, we plated the cells in bulk culture and allowed them to expand for 48 h before determining viable cell numbers. In the presence of S17 + KL + IL-7, there

was a 10-fold expansion of E47$^{+/-}$;GFP BLPs (Fig. 6 A). By contrast, there was only a 1.9-fold expansion of E47$^{-/-}$; EBF BLPs under the same conditions (Fig. 6 A, p < 0.02). In the presence of IL-7 alone, E47$^{+/-}$;GFP cells expanded 6.2-fold, whereas E47$^{-/-}$;EBF BLPs decreased in number by ∼1.9-fold (Fig. 6 A, p < 0.004). Therefore, under either culture condition there was a dramatically impaired ability of the E47$^{-/-}$;EBF BLPs to expand in response to IL-7. Both BLP populations were dependent on IL-7 because removal of IL-7 from the culture media resulted in a decrease in cell numbers and significant apoptosis (unpublished data).

To determine whether the decreased expansion of E47$^{-/-}$; EBF BLPs in S17 + KL + IL-7 was due to reduced proliferation, we examined the cell cycle status of progenitors using BrdU and PI. During a 20-min incubation with BrdU, E47$^{-/-}$;EBF BLPs had ∼30% more cells in the G1 phase of the cell cycle (BrdU$^-$, 2N DNA content) compared with E47$^{+/-}$;GFP cells (59.5 vs. 44.5%; Fig. 6 B). Moreover, ∼30% fewer cells were in the S phase (BrdU$^+$) in cultures of E47$^{-/-}$;EBF BLPs (Fig. 6 B). From these data we conclude that E47$^{-/-}$;EBF BLPs proliferate less well in response to IL-7 than E47$^{+/-}$;GFP BLPs.

Decreased Expression of N-myc in E47$^{-/-}$;EBF BLPs. We considered the possibility that a decrease in E protein activity might affect the expression of the IL-7R on BLPs as IL-7-Rα has previously been suggested to be an E2A-dependent gene (17). However, by Northern blot analysis we found that IL-7Rα mRNA was expressed at equivalent levels in the two populations of cells (Fig. 6 C). In addition, IL-7Rα could be detected on the surface of these cells by flow cytometry (unpublished data). Therefore, the poor proliferative response of E47$^{-/-}$;EBF BLPs to IL-7 is unlikely to be due to the absence of IL-7R expression.

To gain a better understanding of the alterations in E47$^{-/-}$;EBF BLPs that lead to this proliferation defect, we analyzed gene expression patterns in two populations of paired E47$^{+/-}$;GFP and E47$^{-/-}$;EBF BLPs using Affymetrix 430A microarrays. Surprisingly, we found few genes that were consistently reduced by more than twofold in E47$^{-/-}$;EBF cells compared with E47$^{+/-}$;GFP cells (including the B lineage genes present on the chip, although

Table I. *Frequency of CD19$^+$ Cells Responsive to S17 + KL + IL-7 or IL-7 Alone*

| Experiment | Sample | Culture conditions | |
		S17 + KL + IL-7	IL-7
1	E47$^{+/-}$;GFP	1:17	1:43
	E47$^{-/-}$;EBF	1:30	1:280
2	E47$^{+/-}$;GFP	<1:10[a]	1:21
	E47$^{-/-}$;EBF	<1:10[a]	1:204
3	E47$^{+/-}$;GFP	1:20	ND
	E47$^{-/-}$;EBF	1:35	ND

The frequency was determined as the average number of cells per well, where 37% of wells were nonresponding. For each determination, three cell concentrations were plated with a minimum of 48 wells each.
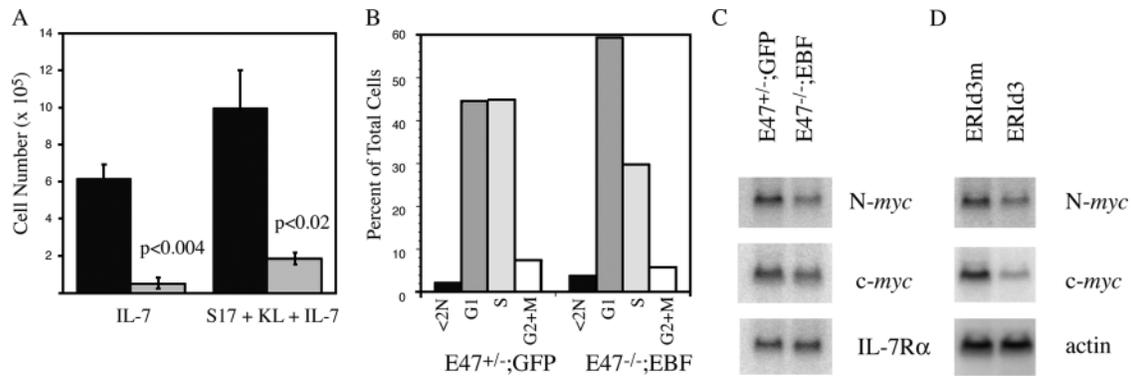[a]All wells were positive when plated at 10 cells/well.

Figure 6. $E47^{-/-}$;EBF BLPs proliferate less well than $E47^{+/-}$;GFP BLPs and have reduced expression of N-*myc*. (A) 10^5 B220$^+$ CD19$^+$ cells from $E47^{+/-}$;GFP (solid bars) and $E47^{-/-}$;EBF (gray bars) cultures were plated in IL-7 alone or S17 + KL + IL-7. 48 h later, the cells were harvested and counted on the hemacytometer. The standard error of three replicate cultures is indicated. The p-values were generated using a Student's *t* test. (B) Cell cycle analysis using BrdU and PI of $E47^{+/-}$;GFP and $E47^{-/-}$;EBF BLPs cultured on S17 + KL + IL-7. The percentage of cells in each phase of the cell cycle is plotted. The data represent one of two independent experiments. (C) Northern blot analysis of RNA extracted from $E47^{+/-}$;GFP and $E47^{-/-}$;EBF BLPs or (D) BLPs expressing ERId3 or ERId3m after treatment with 1 μM 17β-estradiol for 6 h. The probes used are indicated on the right of each panel.

RAG-2 was consistently reduced by twofold). The most notable difference was in expression of N-*myc*, a transcription factor involved in cell proliferation and a known target of IL-7R signaling in BLPs (33). By Northern blot analysis we found that N-*myc* mRNA was consistently decreased by approximately threefold (range 2.8–3.1) in $E47^{-/-}$;EBF cells cultured in S17 + KL + IL-7 (Fig. 6 C). We detected only a marginal (less than twofold) reduction in c-*myc* mRNA in $E47^{-/-}$;EBF BLPs (Fig. 6 C). However, the reduced expression of N-*myc* in $E47^{-/-}$;EBF BLPs could be sufficient to cause the decreased proliferation observed in these cells in response to IL-7.

E Protein Activity Is Required for Expression of N-myc in BLPs. Our previous data demonstrated that E proteins are required for the proliferation of BLPs because ectopic expression of Id3 induces cell cycle arrest in these cells (22). To determine whether N-*myc* expression in BLPs requires E protein activity, we infected FL BLPs with the ERId3 or ERId3m retrovirus to antagonize E protein activity. ERId3- and ERId3m-expressing cells were isolated by sorting for GFP$^+$ cells and were allowed to recover in culture before the addition of 1 μM 17β-estradiol for 6 h and extraction of RNA. By Northern blot analysis we found that expression of ERId3, but not ERId3m, led to a decline in expression of both N-*myc* and c-*myc* mRNA (2.6- and 4.7-fold, respectively; Fig. 6 D). Therefore, optimal N-*myc* and c-*myc* expression in BLPs is E protein dependent. Interestingly, the level of E protein activity in $E47^{-/-}$;EBF BLPs appears to be sufficient to allow expression of c-*myc*, but not optimal expression of N-*myc*. Taken together, our data suggest that the reduced proliferative response of $E47^{-/-}$;EBF BLPs to IL-7 might be due to the inability of these cells to fully activate N-*myc* expression in the absence of E2A proteins.

N-myc Promotes Proliferation of $E47^{-/-}$;EBF BLPs. To test the hypothesis that decreased expression of N-*myc* is responsible for the observed defect in IL-7 responsiveness in $E47^{-/-}$;EBF BLPs, we introduced N-*myc* into these cells and examined their ability to respond to IL-7. Previ-

ous studies have demonstrated that high levels of N-*myc* lead to cell death (34). Consistent with this, we found that a significant percentage of $E47^{+/-}$;GFP and $E47^{-/-}$;EBF BLPs infected with the N-*myc* retrovirus underwent apoptosis within the first 48 h after infection. However, after this time there was an increase in cells with intermediate levels of GFP that were viable and presumably expressing levels of N-*myc* that do not induce apoptosis. Therefore, we used this population of cells to quantify the expansion of N-*myc* or GFP virus–infected cells in the presence of S17 + KL + IL-7 or IL-7 alone (Fig. 7 A). After 48 h of culture, there was an approximate sixfold increase in $E47^{+/-}$; GFP cells in S17 + KL + IL-7 cultures regardless of whether the cells were infected with GFP or N-*myc* virus. In contrast, only a twofold increase in cell numbers was observed in S17 + KL + IL-7 cultures of $E47^{-/-}$;EBF BLPs infected with GFP virus (Fig. 7 A). However, $E47^{-/-}$; EBF BLPs infected with N-*myc*–producing virus underwent a greater expansion resulting in a threefold increase in cell numbers (P < 0.03). Similarly, in the presence of IL-7 alone, N-*myc* did not affect the number of cells recovered from $E47^{+/-}$;GFP cultures, whereas it resulted in a fivefold increase in cell numbers from $E47^{-/-}$;EBF BLPs compared with GFP virus–infected cells (Fig. 7 B; P < 0.001). The rescue of BLP numbers in $E47^{-/-}$;EBF IL-7 cultures was particularly evident upon visual examination because BLPs infected with GFP virus had essentially no viable cells, whereas numerous small colonies could be detected in the N-*myc*–infected cultures. Moreover, we have been able to establish long-term (>2 mo) cultures of $E47^{-/-}$;EBF cells expressing N-*myc* in IL-7, whereas the same cells lacking N-*myc* fail to survive (unpublished data). These data demonstrate that ectopic expression of N-*myc* is sufficient to promote the survival or expansion of $E47^{-/-}$;EBF cells. However, this rescue appears to be critically dependent on the dose of N-*myc*.

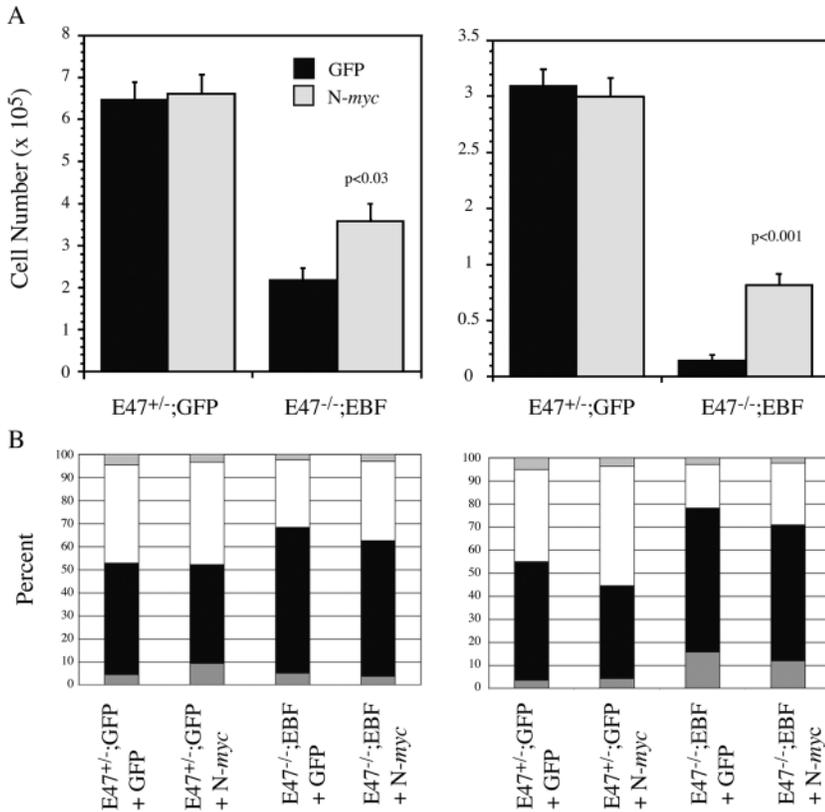To confirm further our observation that N-*myc* promotes proliferation of $E47^{-/-}$;EBF cells, we used BrdU in-

Figure 7. N-*myc* promotes the proliferation of E47⁻ᐟ⁻;EBF BLPs. (A) E47⁺ᐟ⁻;GFP and E47⁻ᐟ⁻; EBF BLPs were infected with GFP (solid bars) or N-*myc* (gray bars)–producing retrovirus and 48 h later, three replicate wells of 10⁵ cells were plated on S17 + KL + IL-7 (right) or IL-7 alone (left). The total number of cells present in each well was determined 48 h later. The p-values were generated using a Student's *t* test. (B) 96 h after infection with GFP or N-*myc* retrovirus, E47⁺ᐟ⁻;GFP and E47⁻ᐟ⁻;EBF BLPs were incubated for 20 min with BrdU and then stained with anti-BrdU and PI. The percent of cells in each phase of the cell cycle was determined by flow cytometry.

corporation and PI staining to determine the percent of cells in each phase of the cell cycle. This analysis revealed that N-*myc* promoted an increase in the percent of E47⁻ᐟ⁻; EBF cells in S phase when cultured on S17 + KL + IL-7 or IL-7 alone as compared with GFP virus–infected cells (32.9 vs. 27% and 21 vs. 14.7%, respectively; Fig. 7, C and D). In contrast, an increase in the percent of BLPs in S phase was observed for E47⁺ᐟ⁻;GFP + N-*myc* cells in IL-7 cultures only (40.6 vs. 39.6% in S17 + KL + IL-7 and 45.7 vs. 35.8% in IL-7 alone). It is currently unclear why this increase in S phase E47⁺ᐟ⁻;GFP + N-*myc* cells did not translate into an increase in cell numbers as was observed for the E47⁻ᐟ⁻;EBF + N-*myc* cells. One possibility is that the increase in cell cycle was balanced by an increase in cell death as this population of cells had a more heterogeneous expression of the N-*myc* retrovirus compared with E47⁻ᐟ⁻; EBF cells (unpublished data). Taken together, our data demonstrate the N-*myc* can promote the ability of E47⁻ᐟ⁻; EBF cells to survive and proliferate in the presence of IL-7.

Discussion

We have demonstrated that the block in B lymphocyte development imposed by the absence of E2A proteins can be overcome by ectopic expression of the E2A target gene EBF. Expression of EBF in E2A-deficient FL progenitors is sufficient to promote the development of B220⁺ CD19⁺ BP1/6C3⁺ cells that express most B lineage genes and undergo VDJ recombination at the IgH locus. The ability of EBF to rescue the development of BLPs from progenitors that lack E2A proteins afforded us the opportunity to examine these cells for additional defects that might be attributed to the reduced level of E proteins. This analysis revealed that E2A proteins are also required for optimal expression of N-*myc*, a target of IL-7 signaling in BLPs that is associated with proliferation (33). In agreement with this finding, E47⁻ᐟ⁻;EBF BLPs had minimal cloning efficiency in IL-7 alone and did not proliferate optimally in response to IL-7 even in the presence of stromal cells and KL. Moreover, this defective response to IL-7 could be restored partially by ectopic expression of N-*myc* in E47⁻ᐟ⁻;EBF BLPs. Therefore, the major function of E2A proteins in B lymphocyte lineage specification is to induce EBF as well as maintain an appropriate context for responsiveness to the cytokine IL-7. Our data also demonstrate that a higher threshold of E protein activity is required for activation of EBF and N-*myc* as compared with other B lineage genes.

A recent study suggested that activation of EBF might be the limiting factor driving B lymphocyte development from common lymphoid progenitors (35). However, the ability of BLPs to develop in the absence of E2A was unexpected because E2A is thought to play an essential role in promoting expression of B lineage–associated genes in cooperation with other B lineage transcription factors including EBF (2, 3, 19, 20, 36). Our data indicate that the low level of alternative E proteins in these cells is sufficient to function in a combinatorial manner with other transcription factors to promote lymphocyte survival and expression

of most B lineage genes. This finding is consistent with the hypothesis that E proteins are largely redundant in function and that E2A proteins specifically are required for B lymphocyte development because E47 is present at much higher levels than other E proteins in BLPs. It is also evident that higher levels of E protein activity are required for the initial induction of EBF than for expression of most other B lineage genes. Whether this requirement is due to the affinity of E proteins for the relevant E-box sites in the EBF regulatory elements, or due to the mechanism of activation of the EBF gene, such as a need for high E-box occupancy for acetylation of chromatin, remains to be determined. Interestingly, the reported EBF promoter contains one high affinity E-box site upstream of two potential Ikaros binding sites, which could function to keep the EBF locus in a repressed chromatin conformation (18, 37). A high level of E protein expression might be required to maintain occupancy of the E-box site in repressed chromatin to ensure activation. Alternatively, there might be additional E-box sites in an EBF enhancer that require high levels to E proteins for maximal binding. In contrast, the mb-1 promoter, which is activated in E47$^{-/-}$;EBF BLPs that have low levels of E protein activity, has binding sites for multiple B lineage transcription factors including EBF, E2A (or E proteins), Oct, and Pax5 (20). Cooperative binding of these factors to the mb-1 promoter may reduce the concentration of E protein required to achieve stable promoter activation. Further studies will be required to determine the mechanism of E protein function in the regulation of the EBF and mb-1 genes.

A recent study by Ikawa et al. (38) identified rare progenitors in the Lin$^-$ fraction of E12$^{-/-}$ BM that were able to form stable cell lines in vitro. These E12$^{-/-}$ lines expressed a number of B lineage genes at low levels but remained capable of multilineage hematopoietic reconstitution after transfer into lethally irradiated mice. In contrast to this study, we did not find evidence of in vitro expansion of cells expressing B lineage genes from the Lin$^-$ fraction of E47$^{-/-}$ FL. We were only able to detect cells expressing B lineage genes after expression of EBF, and not Pax5, in E47$^{-/-}$ FL cells. Moreover, expression of EBF in E47$^{-/-}$ FL is sufficient to promote B lineage commitment because the cells began to express CD19 and Pax5 and were unable to differentiate into myeloid cells in the presence of M-CSF. The observed differences between the Ikawa study and ours might be the results of the origin of the progenitors (FL vs. BM), the culture conditions used, or the genotype of the mice (E47$^{-/-}$ vs. E12$^{-/-}$). However, our results clearly indicate that EBF is the essential target of E2A proteins required for B lymphopoiesis.

Our previous studies demonstrated that inhibition of E protein activity in BLPs by ectopic expression of Id3 induces growth arrest and apoptosis (22). Interestingly, the low level of E protein activity remaining in E47$^{-/-}$;EBF BLPs is sufficient to sustain the viability of these cells in the absence of E2A. However, E47$^{-/-}$;EBF BLPs are exquisitely sensitive to the expression of Id3, further confirming

the need for HEB and/or E2-2 to promote B lymphopoiesis in the absence of E2A. Regardless, HEB and E2-2 are not sufficient to promote all aspects of B lymphopoiesis in E47$^{-/-}$;EBF cells as these cells have reduced N-*myc* mRNA levels and do not proliferate well in response to IL-7. IL-7 has been shown to maintain both N-*myc* and c-*myc* expression in BLPs, and the overall level of myc proteins is a major factor driving expansion of these cells (33, 39). Remarkably, we were able to increase the response of E47$^{-/-}$; EBF cells to IL-7 by ectopic expression of N-*myc*, although this response appears to be critically dependent on the level of N-*myc* expression. Our findings indicate that the reduced expression of N-*myc* is likely to be the cause of the poor proliferative response of E47$^{-/-}$;EBF BLPs to IL-7. Interestingly, we found that optimal expression of both N-*myc* and c-*myc* depends on E protein activity in BLPs because both were reduced in cells expressing ERId3. This observation indicates that the low level of N-*myc* in E47$^{-/-}$; EBF cells might be the direct consequence of the absence of E2A and not a consequence of growth arrest, which would also cause a decline in c-*myc* expression. The reason that c-*myc* expression was less affected in E47$^{-/-}$;EBF BLPs is not clear, but suggests that distinct thresholds of E protein activity are required for optimal expression of c-*myc* and N-*myc* mRNA. Our observations place N-*myc* into a unique class of B lineage genes, including EBF, which require high levels of E protein activity for their maximal expression. Our data do not, however, require that N-*myc* be a direct transcriptional target of E proteins. The possibility remains that E proteins regulate the expression of a distinct gene that is required for the induction of N-*myc* in response to IL-7.

IL-7R signals have also been implicated in the regulation of V gene usage at the IgH locus (40, 41). In the absence of IL-7Rα, V gene segments of the V$_{J558}$ family are rearranged less efficiently than V$_{7183}$ family members. We found that E47$^{-/-}$;EBF BLPs had a slight alteration in V gene usage. The V$_{J558}$ family of V gene segments was used with slightly less efficiency than V$_{7183}$ and V$_{Q52}$ gene segments (Fig. 3 D). It is possible that the decreased rearrangement of V$_{J558}$ is due to reduced IL-7R–mediated activation of N-*myc* in E47$^{-/-}$;EBF BLPs. However, an alternative and more likely possibility is that E protein activity has a stronger influence on the activation of promoters upstream of V$_{J558}$ gene segments than V$_{7183}$ or V$_{Q52}$ gene segments. The hypothesis that E proteins may influence expression of a subset of V gene segments is consistent with the differential usage of Vκ and Vλ gene segments reported in nonlymphoid cells transfected with E2A or EBF (42).

The IL-7R signaling pathway has also been implicated in the induction of the Pax5 gene (40). E47$^{-/-}$;EBF BLPs express Pax5 at levels comparable to E47$^{+/-}$;GFP cells, indicating that the IL-7R signaling pathway leading to the induction of Pax5 is not affected by the absence of E2A. Alternatively, ectopic expression of EBF might be able to induce Pax5 expression even in the absence of the IL-7–derived signal. EBF can bind to a sequence in the region 5′

of the Pax5 gene and induces expression of Pax5 in the 70Z/3 macrophage cell line, suggesting that Pax5 might be a direct target of EBF (17, 21). Interestingly, the 70Z/3 macrophage cell line has no detectable E-box binding proteins, indicating that the induction of Pax5 in these cells may not require E proteins directly (17). Therefore, EBF might be the major B lineage transcription factor driving expression of Pax5. Taken together, our data demonstrate that E2A proteins play an essential function in coordinating both the differentiation and expansion of B lineage progenitors by induction of EBF and N-myc.

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