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Cloning and Characterization of a Promoter Flanking the Early B Cell Factor (EBF) Gene Indicates Roles for E-Proteins and Autoregulation in the Control of EBF Expression[1]

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The early B cell factor (EBF) is a transcription factor shown crucial for the development of B lymphocytes. The protein is expressed from the earliest stages of B cell development until the mature B cell stage, but the control elements responsible for the regulation of the gene are unknown. In this study, we report of the identification of a promoter region flanking the *EBF* gene. Several transcription start sites were identified by primer extension analysis in a region ~3.1 kb from the predicted ATG. Transient transfections revealed that this region was able to stimulate transcription of a reporter gene in B lymphoid and to a lesser extent, myeloid cells, but not in a pre-T cell line. The promoter was also able to functionally interact with E47, suggesting that the EBF gene may be a direct target for activation by E-proteins. In addition, functional binding of EBF to its own promoter was confirmed by EMSA and transfection assays indicating that the EBF protein may be involved in an autoregulatory loop. Finally, a tissue-restricted factor was able to bind an upstream regulatory region in B-lineage cells, further supporting the idea that the cloned promoter participates in the regulation of stage and lineage specific expression of the EBF gene. ***The Journal of Immunology*****, 2002, 169: 261–270.**

The development of specific hemopoietic lineages from common precursors in the bone marrow is a complex process demanding a highly ordered regulation of stage- and lineage-specific gene expression. The development of a B lymphocyte can be divided into multiple stages that may be characterized by the expression of genes involved in Ig rearrangements and signaling, as well as by the actual recombination status of the Ig genes (1–3). This process has been shown to be critically dependent of a number of transcription factors (4–6) including Ikaros (7, 8), B cell-specific activator protein (BSAP)[3] (9, 10), the E2A-encoded E47/E12 proteins (11, 12), and early B cell factor (EBF)/Olf-1 (13). EBF has been shown essential specifically for B cell development since mice lacking this protein, due to disruption of the coding gene, are unable to develop B lymphoid cells while the development of all other hemopoietic lineages appear normal (13). The bone marrow of these mice contain cells expressing B220 and CD43 (13) suggesting that they belong to fraction A according to Hardy et al. (1), but no B lineage cells of subsequent differentiation stages. The dramatic phenotype of EBF-deficient mice suggests this protein to be involved in the regulation of genes crucial for B cell development. However, even though several potential target genes are identified (14–19), the exact cause of the impaired B lymphocyte development in EBF-deficient mice remains unclear.

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[3] Abbreviations used in this paper: BSAP, B cell-specific activator protein; EBF, early B cell factor; RACE, rapid amplification of cDNA ends; M-MuLV-RT, Moloney murine leukemia virus reverse transcriptase; EPBP, EBF promoter binding protein.

EBF is expressed in B lymphocytes, adipose tissue (20), and stromal cell lines (M. Sigvardsson, unpublished observations), as well as in neural cells of different origins (21, 22). The rather broad overall expression pattern is contrasted by an apparently restricted expression in hemopoiesis where EBF only appear to be expressed in B lineage cells (20, 23). This expression pattern, in combination with the large number of B cell-restricted target genes (14–19), and the phenotype of EBF-deficient mice (13) indicate that EBF may be of key importance for the specification of the B lineage. Thus, the resolution of how the EBF gene is transcriptionally controlled might give extended insight into how lineage commitment is achieved in the bone marrow.

The EBF gene has been suggested to be a target for the basic-helix-loop-helix E-protein E12, encoded by the E2A gene (24), because stable ectopic expression of this protein in de-differentiated 70Z/3 pre-B cells resulted in the production of EBF transcripts (25). To directly investigate how the EBF gene is transcriptionally controlled, we made 5′ rapid amplification of cDNA end (RACE) experiments and compared the obtained cDNAs with genomic DNA encompassing the 5′ end of the EBF gene. This resulted in the cloning of an alternatively spliced EBF message and a novel exon located ~3.1 kb from the predicted ATG. Primer extension analysis suggested that transcription was initiated at multiple sites within this region and two major start sites could be mapped to potential initiator elements. Cloning of genomic DNA including these start sites in front of a reporter gene revealed that this region composed a promoter element able to stimulate initiation of transcription. The promoter harbored binding sites for both EBF and E47, indicating that the *EBF* gene is controlled by autoregulation and is a direct target for activation by E47 or possibly other E-proteins.

Materials and Methods

Isolation of primary cells

$B220^+$ cells were obtained by homogenization of spleens from C57BL/6 mice, followed by filtering through a 70-μm mesh. The

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$B220^+$ cells was then purified on an anti-B220 MACS column (Miltenyi Biotec, Auburn, CA).

RT-PCR

RNA was prepared from cells using TRIzol (Life Technologies, Grand Island, NY), and cDNA was generated by annealing 1 μg of total RNA to 0.5 μg of random hexamers in 10 μl diethyl pyrocarbonate-treated water. RT reactions were performed with 200 U of Moloney murine leukemia virus reverse transcriptase (M-MuLV-RT) (Boehringer Mannheim, Bromma, Sweden) in accordance with the manufacturer's recommendations. One-twentieth of the RT reaction was used in the PCR assays. PCR was performed with 1 U of *Taq* polymerase (Life Technologies) in the manufacturer's buffer supplemented with 0.2 mM dNTP, in a total volume of 25 μl. GADPH was amplified by 28 cycles (94°C, 30 s, 55°C, 30 s, and 72°C, 30 s) while 30 cycles were used to amplify EBF cDNA (94°C, 30 s, 61°C, 30 s, and 72°C, 30 s). Primers were added to a final concentration of 1 mM. The PCR products were blotted onto Hybond N^+ nylon membranes (Amersham, Uppsala, Sweden) using capillary blotting with 0.4 M NaOH. Membranes were prehybridized in 5× Denhart's solution, 6× SSC, 0.1% SDS, and 50 μg/ml salmon sperm DNA, at 57°C for 90 min and hybridized with α-^{32}P-labeled random primed cDNAs at 65°C in the same solution. Membranes were washed at room temperature two times in 2× SSC supplemented with 0.1% SDS for 15 min.

Oligonucleotides used for RT-PCR were GADPH sense 5′-CACAGG ACTAGAACACCTGC; GADPH antisense 5′-GCTGGTGAAAAGGA CCTCT; exon 1 sense 5′-GTTCGGCAGTCCCAACACGCATCC; and exon 1 antisense 5′-CCAGCGGCTCTTCCTTCATAC.

Tissue culture conditions

All cells were grown in RPMI medium supplemented with 7.5% FCS, 10 mM HEPES, 2 mM pyruvate, 50 μM 2-ME, and 50 μg gentamicin/ml (complete RPMI medium; all purchased from Life Technologies, Täby, Sweden) at 37°C and 5% CO_2.

Transient transfections and luciferase assays

Lymphoid cells were washed twice in TBS (140 mM NaCl, 5 mM KCl, 25 mM Tris, pH 7.4, 0.6 mM Na_2HPO_4, 0.5 mM $MgCl_2$, 0.7 mM $CaCl_2$). Transfection was performed by incubating 2.5×10^6 cells for 30 min at 20°C in 0.65-ml DEAE-dextran (0.7 mg/ml TBS; Pharmacia, Uppsala, Sweden) containing 2 μg reporter gene construct. The transfected cells were thereafter washed once in TBS and then cultured in 5 ml of complete RPMI medium in six-well plates for 48 h. Preparation of protein extracts and luciferase assays were performed with a dual-luciferase reporter assay system (Promega, Madison, WI) using 20% of the total protein extract. The obtained luciferase activity was normalized against the activity of a co-transfected (0.25 μg) CMV-controlled *Renilla* luciferase reporter gene.

HeLa cells (500,000) were washed once with serum-free medium (OPTIMEM; Life Technologies) and 800 μl of the medium was added for transfection. Lipofectin (5 μl; Life Technologies) was diluted in 100 μl of serum free medium, incubated for 45 min in room temperature and mixed with the DNA diluted in 100 μl medium. The mixture was incubated for 25 min and the combined volume of 200 μl was added to the cells. The cells were then incubated in a CO_2 incubator at 37°C for 12 h, after which the transfection medium was removed and replaced by RPMI supplemented with 10% FCS. The cells were harvested after 40 h and protein extracts were prepared directly in the 24-well plates by adding 80 μl of cell lysis buffer (Promega, Falkenberg, Sweden). The luciferase assay was conducted using 20 μl of the obtained extracts and 200 μl of Luciferase assay reagent (Promega).

Phage library and cloning of genomic DNA

To obtain genomic DNA encompassing the EBF gene, a λFix library containing genomic DNA inserts from I29 mouse liver was used. PFU (600,000) were plated on 12 14-cm LM-agar plates, nitrocellulose filters were lifted, and the library was screened by hybridization of a random primed ^{32}P-labeled cDNA probe encoding the mouse EBF protein in 5× Denhart's solution, 6× SSC, 0.1% SDS, and 50 μg/ml salmon sperm DNA, at 65°C for 16 h. Membranes were washed twice at room temperature in 2× SSC supplemented with 0.1% SDS for 15 min and twice in a solution containing 0.1× SSC and 0.1% SDS. Phage DNA was digested with *Sal*I, and the inserts of genomic EBF DNA were ligated into the *Sal*I site of a pGEM3Z plasmid.

5′ RACE, primer extension, and Northern blot analysis

Poly(A) + RNA was obtained by purification of total RNA, prepared by TRIzol extraction as above, with a micro mRNA preparation kit (Amersham) according to the manufacturer's instructions. mRNA (5 μg) was then used for cDNA synthesis and RACE reaction using a RACE analysis kit (Life Technologies), according to the manufacturer's protocol. Briefly, an oligonucleotide primer located ~250 bp into the cloned EBF gene, was annealed to the mRNA and first strand synthesis was performed with Expand M-MuLV-RT (Roche, Basel, Switzerland). The product was purified and the cDNA was modified by TdT-mediated addition of a 5′ oligo G tail. The RACE product was then amplified by PCR using a second nested EBF specific primer and an oligo C primer. The obtained PCR product was cloned into a pGEM3Z plasmid and sequenced. RACE primer 1, 5′-AGT GGAAGAAGTTGGATTTCCG; nested RACE primer, 5′-GAAGGCG GCTGCTTCTCAAAGTG.

Primer extension analysis was performed using 5 μg poly(A) RNA to which a ^{32}P-labeled oligonucleotide was annealed in 30 μl 40 mM PIPES, pH 7.4, 0.4 M NaCl, 1 mM EDTA and 80% deionized formamide. The mixture was incubated at 85°C for 10 min followed by an overnight incubation at 42°C. The obtained hybrid was precipitated by the addition of 70 μl of diethyl pyrocarbonate-treated H_2O and 250 μl ethanol. After centrifugation, the pellet was washed twice with 80% ethanol. The dry pellet was then redissolved in 20 μl RT-reaction buffer and extension was achieved by the addition of 5 U M-MuLV-RT (Boehringer Mannheim). The obtained extension products were extracted with phenol and chisam, precipitated by ethanol precipitation, redissolved in formamide, and loaded on a 7% polyacrylamide urea sequencing gel. The sequence ladders were obtained by T7-DNA polymerase primer extension in the presence of dideoxynucleotides. TS1 was defined based on size and alignment to a sequence ladder generated by primer extension using the cloned genomic DNA as template (see Fig. 2*B*). TS2 was defined by direct comparison to a sequence ladder obtained by extension of the same primer as used for primer extension with genomic DNA as template (see Fig. 2*B*). Primer extension 1, 5′-CAAGGCCCTCTGAGAGCTTCTGG; primer extension 2, 5′-CCGAAGCCAGGCCACTATCAAGG.

Northern blot analysis was performed using 5 μg of poly(A)-positive RNA prepared as above. The RNA was separated in a 1.2% PIPES/formaldehyde agarose gel and blotted onto a Hybond N^+ membrane by capillary transfer in 2× SSC. After UV cross linkage, the membranes were prehybridized in 6× SSC, 0.1% SDS, and 5× Denhart's solution followed by hybridization overnight of a random primed 300-bp PCR product from exon 1 at 55°C. The membrane was then washed twice with 2× SSC, 0.1% SDS, and twice with 0.1× SSC, 0.1%SDS at room temperature.

Protein extracts, EMSA

Nuclear extracts were prepared according to Schreiber et al. (26). DNA probes were labeled with [γ-^{32}P]ATP by incubation with T4 polynucleotide kinase (Boehringer Mannheim), annealed, and purified on a 5% polyacrylamide Tris-borate-EDTA gel. Nuclear extract, or in vitro transcribed-translated protein, was incubated with labeled probe (20,000 cpm, 3 fmol) for 30 min at room temperature in binding buffer (10 mM HEPES, pH 7.9, 70 mM KCl, 1 mM DTT, 1 mM EDTA, 2.5 mM $MgCl_2$) with 0.75 μg poly(dI/dC) (Pharmacia). DNA competitors were added 10 min before the addition of the DNA probe. The samples were separated on a 6% polyacrylamide Tris-borate-EDTA gel, which was dried and subjected to autoradiography. Competitors were added at molar excesses indicated in the respective figure legends.

Oligonucleotides used for electrophoretic mobility shift assays were as follows: Mb-1 EBF sense, 5′-GAGAGAGACTCAAGGGAATTGTGG; Mb-1 EBF antisense, 5′-CCACAATTCCCTTGAGTCTCTCTC; λ5E sense, 5′-TCTTGTTCCATGGGGCAGGTGTTCAGTTGCTCTCTACGGC; λ5E antisense, 5′-GCCGTAGAGAGCAACTGAACACCTGCCCCATG GAACAAGA; OCT sense, 5′-TTCATTGATTTGCATCGCATGAGAC GCTAACATCGTACGTTC; OCT antisense, 5′-GAACGTACGATGTT AGCGTCTCATGCGATGCAAATCAATGAA; EBF promoter E-box sense, 5′-CGTTTCCTCACCTGTACAATGG; EBF promoter E-box antisense, 5′-CCATTGTACAGGTGAGGAAACG; EBF promoter EBF site sense, 5′-TCTGGGACGATCCCGGGGGAAACCCGAGAGAGGCCTCAGCT CGT; EBF promoter EBF site antisense, 5′-ACGAGCTGAGGCCTC TCTCGGGTTTCCCCCGGGATCGTCCCAGA; EBFP E-BOXM sense, 5′-CGTTTCCTCTGCAGTACAATGGGAGTG; EBFP EBOXM antisense, 5′-CATTGTACTGCAGAGGAAACGGAGGG; EBFP EBFM sense, 5′-GGACGATCCATATGGAAACCCGAGAGAGG; EBFP EBFM antisense, 5′-GGTTTCCATATGGATCGTCCCAGACAAT; EBF promoter −536 to −483 sense, 5′-GAATTCTAGTGGTGTTGAATAGCCCTTAGCT GCCTCTGCCGGGTGGAGGTTTGACT; EBF promoter −536 to −483 antisense, 5′-AGTCAAACCTCCACCCGGCAGAGGCAGCTAAGGGCTAT TCAAC ACCACTAGAATTC; M1 sense, 5′-GAATTCTAGTGGTGTTGA ATACAGCGTCACTGCCTCTGCCGGGTGGAGGT; M1 antisense, 5′-AGT CAAACCTCCACCCGGCAGAGGCAGTGACGCTGTATTCAACACCACT

A; M2 sense, 5′-GAATTCTACTCAGTTACGATAGCCCTTAGCTGCCTCT GCCGGGTGGAGGT; M2 antisense, 5′-AGTCAAACCTCCACCCGGCAGA GGCAGCTAAGGGCTATCGTAACTGA GTA; M3 sense, 5′-GAATTCTAC TCATGTTGAATAGCCCTTAGCTGCCTCTGCCGGGTGGAGGT; M3 antisense, 5′-ACCTCCACCCGGCAGAGGCAGCTAAGGGCTATTCAACATG AGTAGAATTC; M4 sense, GAATTCTAGTGGTGTACGATAGCCCTTAG CTGCCTAGGT; M4 antisense, ACCTCCACCCGGCAGAGGCAGCT AAG GGCTATCGTACACCACTAGAATTC.

Abs used in EMSA analysis were either purchased from Santa Cruz Biotechnology (Santa Cruz, CA); anti-*myc* 9E10 or anti E47 (N-649) were directed against the E47-specific DNA binding and dimerization domain of E47, therefore abolishing DNA binding activity. The anti-EBF rabbit sera was raised against a peptide from the C-terminal region of EBF (R. Gisler and M. Sigvardsson, unpublished observations).

Plasmids and constructs

The EBF and E47 expression plasmids (16, 27) was based on the eukaryotic expression vector cDNA3 (Invitrogen, Leek, The Netherlands) which places the inserted cDNA under the control of a CMV promoter. The EBF promoter reporter plasmids were based on the luciferase encoding vector pGl3-basic (Promega). The full-length promoter construct was obtained by the initial cloning of a PCR product spanning the region from +299 bp 3′ of the TS2 transcription initiation site up to the *Eco*RI site, generated by 20 cycles of high fidelity PCR (Boehringer Mannheim) using primer extension primer 1 and a T7 promoter primer with a subcloned *Eco*RI fragment as template. The obtained fragment was cloned into the *Sma*I site of pGl3 to yield the ΔRI reporter plasmid. The construct was verified by sequencing, digested with *Eco*RI, and extended by ligation of a 6-kb *Eco*RI fragment from genomic DNA. The extended promoter was then digested with *Sac*I and re-ligated, resulting in the Δ*Sac*I plasmid. The remaining 5′ deletions were obtained by nuclease digestions using the Erase-a-Base system (Promega) after digestion of the full-length plasmid with *Nhe*I followed blunting with thioDNTPs and Klenow enzyme. The plasmid was then redigested with *Mlu*I to create a nuclease-sensitive 5′ overhang. The sizes of the obtained promoter fragments were estimated by restriction enzyme analysis and the Δ1 deletion by sequencing. The ΔP construct was obtained by cloning of a PCR fragment generated from the ΔRI plasmid using a RV3 (Promega) primer and an antisense E-box oligonucleotide (see above), into the *Sma*I site of pGl3. The construct was verified by sequencing. Point mutations in the EBF promoter E-box and EBF sites were introduced by PCR amplification of the 5′ part of the promoters using antisense primers where the binding sites had been disrupted by the insertion of restriction sites (*Nde*I in the EBF site and *Pst*I in the E-box) and the RV3 primer, with the ΔRI construct as template. The 5′ end of the promoters were then cloned into pGl3,redigested, and ligated to the 3′ part of the promoter amplified by use of mutated sense oligonucleotides and Gl-2 primers with the ΔRI promoter as template. The EBF/E-box double mutant promoters were obtained by PCR amplification of the E-box mutated reporter plasmid with the EBF site-mutated antisense oligonucleotide and a RV3. The obtained fragment was digested with *Nde*I and ligated into the EBFM plasmid. All mutations were verified by sequencing.

EBFp (+299) antisense, 5′-CAAAGGCCCTCTGAGAGCTTCTGG; EBFP E-BOXM sense, 5′-CGTTTCCTCTGCAGTACAATGGGAGTG; EBFP EBOXM antisense, 5′-CATTGTACTGCAGAGGAAACGGAGGG; EBFP EBFM sense, 5′-GGACGATCCATATGGAAACCCGAGAGAGG; EBFP EBFM antisense, 5′-GGTTTCCATATGGATCGTCCCAGACAAT.

The TATA box reporter plasmid was a kind gift from Professor T. Leanderson and consists of a SP6 κ promoter TATA-box cloned into the polylinker of pGl3 (28).

In vitro transcription and translation

Recombinant proteins were generated by coupled in vitro transcription-translation by using a reticulocyte lysate kit (Promega). Reaction mix (0.5 of 25 μl) was used for EMSAs.

Results

The EBF *gene contains a novel noncoding exon 3.1 kb 5′ of the coding gene*

The published EBF cDNA corresponds to a message of 2.3 kb, while Northern blot analysis from pre-B or B cell lines indicate the presence of several messages ranging from 2.7 to 5.6 kb in size (20). This suggests that the actual transcription initiation site and promoter of the EBF gene might be located at a rather large distance from the defined translation start codon. To obtain a 5′ end of the mRNA, we performed 5′ RACE analysis using poly(A) RNA from the EBF expressing pre-B cell line 70Z/3 and oligonucleotides positioned ~200 bp 3′ of the ATG in the published cDNA (20). This resulted in a number of PCR products containing 5′ parts of the EBF mRNA. Several of these represented the continuation of the published cDNA presenting a perfect correlation with the genomic DNA while others presented a differentially spliced 5′ end. This mRNA was spliced to a site just 3′ of the predicted ATG, deleting this from the message, extending into a new 5′ exon (Fig. 1*A*). The novel exon contained two potential translation start codons, but these would produce short peptides due to in-frame stop codons and therefore, it is more likely that translation is initiated at the second ATG in the published EBF cDNA resulting in the truncation of 14 aa (Fig. 1, *A* and *C*). The splicing occurred between a consensus splice donor at the 3′ end of the new exon and a splice acceptor site in the coding region of EBF (Fig. 1*C*) suggesting that the EBF mRNA can be partially spliced. The expression of this splice form was also verified by RT-PCR analysis using an anti-sense primer located downstream of the alternative splice site and a sense primer in the newly defined exon. The resulting PCR product was then hybridized to a probe covering this new exon. The splice form was detected both in 70Z/3 cells and in primary sorted splenic B cells, but not in EBF nonexpressing BaF/3 cells (16) (Fig. 1*B*). The cDNA analysis was extended to include different primer combinations hybridizing to the region between the ATG and the novel exon 1. This resulted in a large number of differently sized PCR products indicating that this region could give rise to a large number of differently spliced 5′ regions containing the novel exon 1 (data not shown). Several attempts to obtain a longer message by nested 5′RACE based on primers in the novel exon were largely in vain, since of ~40 cloned PCR products examined, all but one ended in the same region as the cDNA defined in the initial RACE analysis (data not shown). To further investigate this, we hybridized a probe spanning the novel exon to a Northern Blot of poly(A)-positive 70Z/3 RNA (Fig. 1*B*). Using the 16S and 23S RNAs from an ethidium bromide-stained parallel lane of total RNA as m.w. marker, we obtained data indicating that the novel exon hybridized to a number of transcripts ranging from ~3 to 5.5 kb. Thus, these data support that the cloned cDNAs represent the 5′ end of the mRNA and that the larger EBF messages are obtained by alternative splicing of a message extending from the novel exon into the coding region.

To localize the new exon in the context of the EBF gene and to identify a promoter, we used a λFix phage-library containing genomic DNA inserts from BALB/c liver. PFU (600,000) were plated and screened using a random-primed mouse EBF cDNA probe. This resulted in 18 positive clones of which two were able to hybridize with a probe covering the 5′ region of the EBF cDNA. Southern blot analysis of these phages suggested that one of them carried a 14-kb insert covering a region of ~8 kb upstream of the predicted EBF ATG and 6 kb 3′ of the same region and was thus selected for further analysis and sequencing. The new exon could be identified in a region of the genomic DNA that restriction enzyme analysis suggested to be located ~3.1 kb from the previously defined ATG (20) (Fig. 1*C*).

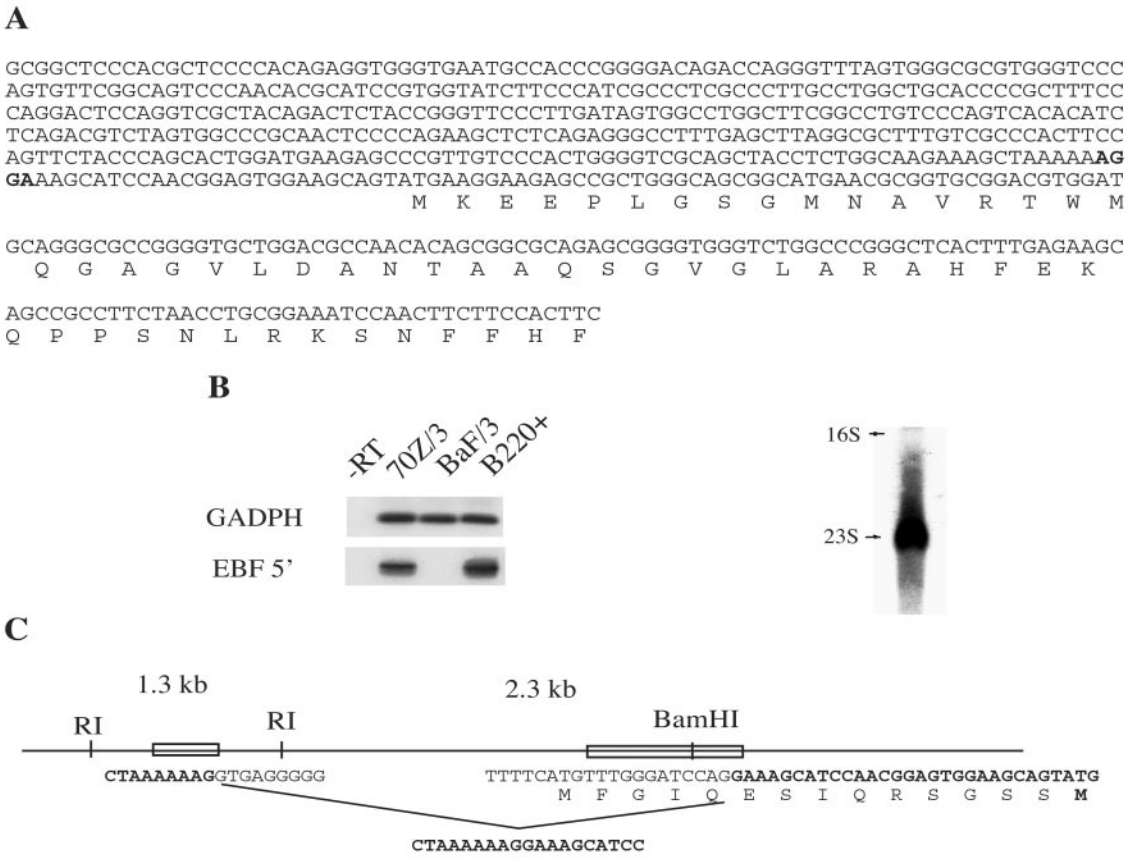

FIGURE 1. 5′ RACE analysis reveals an alternative splice form of EBF encoded by a novel exon located 3.1 kb 5′ of the translation initiation start. *A*, cDNA sequence and translation product from the alternatively spliced EBF RNA. The splice junction is indicated by bold letters. *B*, An autoradiogram with PCR amplification products of the 5′ region of EBF mRNA from the indicated cells hybridized with a random primed DNA probe covering the novel EBF exon. The right part of the panel displays a Northern Blot analysis hybridizing a probe covering the novel exon to poly(A)-selected 70Z/3 RNA. The migration of the 16S and 23S rRNAs are indicated. *C*, A schematic drawing of the 5′ part of the EBF gene including a partial restriction map and splice junctions used in the EBF 5′ splice form. The region and the encoded amino acids deleted from the previously published 5′ end (20) are indicated and the actual ATG for this splice-form is indicated by bold letter M.

The 5′ region of the EBF *gene contains a tissue-restricted promoter*

The results of the RACE analyses suggested that transcription was initiated just 5′ of the novel exon 1 and that this region would contain a promoter region. Sequence analysis of the region 5′ of the new exon (Fig. 2*A*) revealed that the GC content was 67% including 20 CG base combinations indicative of CpG islands known to be involved in transcriptional regulation of genes (29, 30). No obvious TATA box was present in the presumed region of transcription initiation, but several potential initiator elements were found. A computer aided search using the Transfac-Matinspector V2.2 program revealed several potential binding sites for transcription factors including GATA-proteins, NF-κB, AP4, and *ets*-proteins. The analysis also suggested the existence of two perfect binding sites for Ikaros proteins (31) and an E-protein binding E-box (32–34). The region did also contain a potential binding site for EBF itself with an 11 of 14 bp match to the consensus binding site (35).

To identify sites of transcription initiation, we made a primer extension analysis using two different primers located in the novel EBF exon 1. No extension products were obtained using tRNA, while poly(A) + RNA from the pre-B cell line 70Z/3 resulted in several products including three major species (transcription start sites, TS1, 2, and 3; Fig. 2*B*). Of these, site 1 and 3 could not be detected using mRNA from the EBF-negative WEHI3 cells while potential start site 2 was detected at lower levels also in these cells. Thus, to verify the identity of this site, we made a second round of primer extensions using another oligonucleotide located 5′ of the first. This analysis verified the existence of start site 2 because this reduced the background levels obtained in from the WEHI3 cells even further (Fig. 2*B*). Exact start sites were defined by alignment to sequence ladders from genomic DNA and revealed that they displayed similarities to potential initiator (Inr) core sequences ($PyPyA^{+1}NTPyPy$) (36), having six of seven bases match to TS1 and five of seven to TS2 (Fig. 2*A*). Both TS1 and TS2 were also defined to the adenosine nucleotide being the most common start site for Inr-initiated transcription (36). The size of TS3 hindered us from defining the exact start site for this transcript but its presence indicates that transcription also is initiated further 5′ of TS1 and TS2.

To investigate the functional properties of the region 5′ of the transcription initiation site, we cloned a 6-kb fragment including the putative promoter region down to nucleotide +299, as counted from the TS2 start site (Fig. 2*A*), into a pGl3 luciferase reporter vector. Serial 5′ deletions were then obtained by exonuclease III or restriction enzyme digestion and the resulting reporter constructs were transiently transfected into 70Z/3 pre-B cells (Fig. 3*A*). A reporter construct containing 483 bp upstream of TS2 (Δ1) displayed an activity seven times what could be observed after transfection of the same reporter gene under the control of a TATA-box

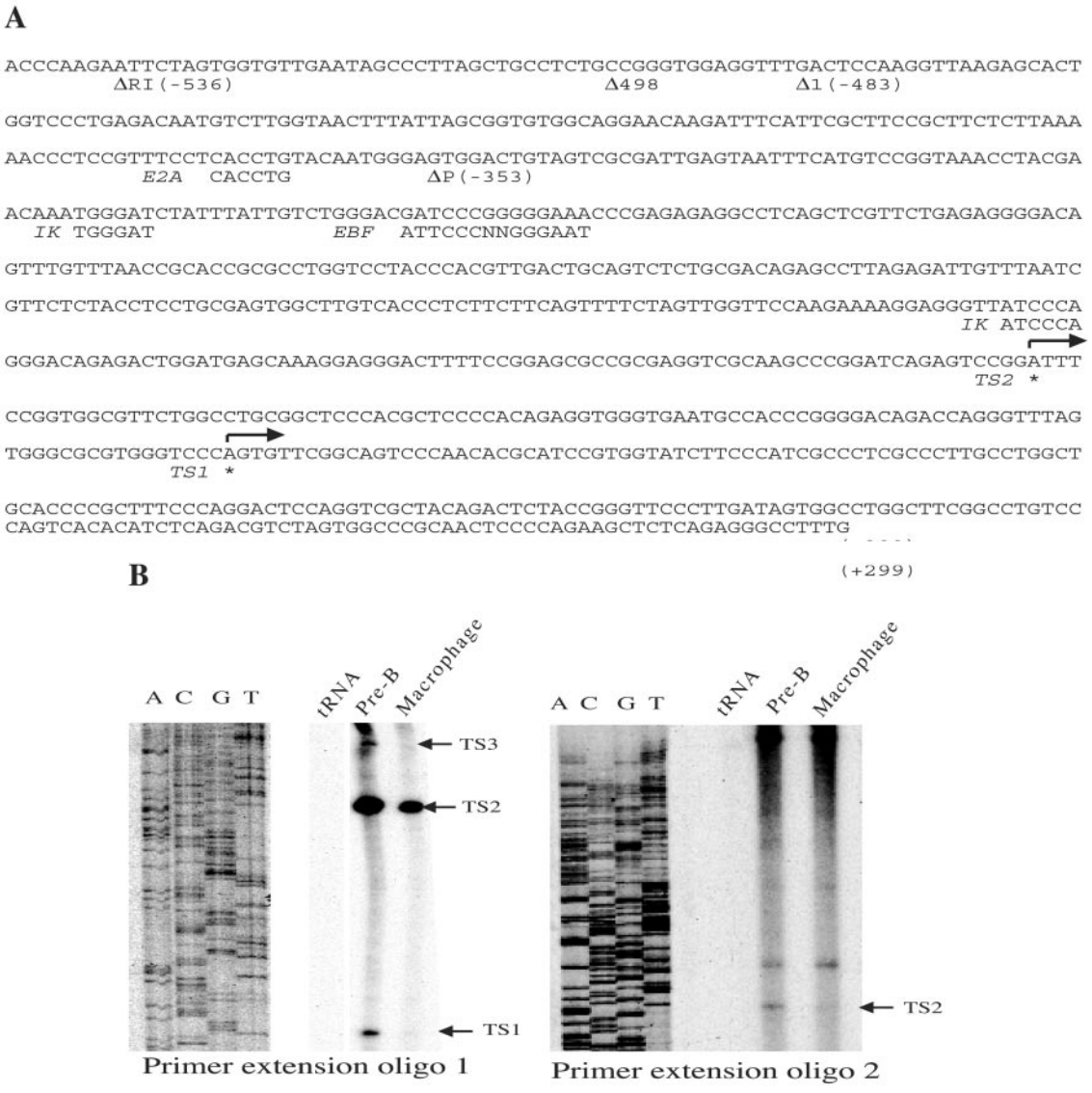

FIGURE 2. Transcription is initiated in the region 5′ of the novel EBF exon. *A,* A sequence analysis of the region 5′ of the cloned EBF splice-form. Transcription start sites defined by primer extension are indicated by TS (arrow and asterisk). Potential binding sites for some transcription factors are indicated and their consensus sequences are aligned to the *EBF* 5′ region. The nucleotide numbering refers to the promoter constructs in Fig. 3. *B,* Two primer extension analyses using different primers and either 5 μg of yeast tRNA, 5 μg 70Z/3 pre-B cell, or WEHI3 macrophage poly(A) RNA. The sequence ladders were obtained by extension by the same primers as used for RNA detection, but with genomic EBF DNA as template. The major pre-B cell-restricted products obtained are denoted TS1–3.

alone, suggesting that the defined region indeed contains a promoter element. This activity was further increased to 8.5 when another 15 bp (Δ498), or 20-fold when another 53 bp (ΔRI), was added to the promoter suggesting that the 5′ part of this region contained additional positive control elements. A comparable activity was observed after the addition of another 450 bp up to the *Sac*I site (ΔSac). Further extension of the promoter region resulted in a gradual loss of functional activity indicating that no more major positive control elements were to be found in this region (data not shown). We also generated a promoter (ΔP) lacking the presumed transcriptional start sites but contained the region from the E-box (−353) to the *Eco*RI site (−536). The transfection of this reporter construct resulted in a functional activity marginally above that obtained with a TATA-box alone, suggesting that the upstream region by itself was unable to stimulate expression of the reporter gene and the transcription is initiated in the region defined by the primer extension analysis. Thus, the region 5′ of the EBF gene do contain a promoter capable to stimulate transcription of a reporter gene in a pre-B cell line.

To investigate the stage and tissue specificity of the promoter, we transiently transfected a set of cell lines representing different B cell developmental stages or other cell lineages with the EBF promoter reporter construct that resulted in the highest functional activity in the 70Z/3 cells (ΔRI) (Fig. 3*B*). Transfection into BaF/3 cells, representing a non-EBF expressing pro-B cell (16), revealed that the promoter was active at a level 15-times that of a TATA-box reporter. When the same reporter genes were transfected into the EBF expressing pre-B cell lines 70Z/3 and 230–238, this resulted in 20- and 17-fold higher activity then the TATA-box. Transfection of the EBF promoter reporter into WEHI231 and K46 cells, representing mature B cells expressing lower levels of EBF (20), resulted in a 10- and 9-fold higher activity than the TATA reporter. When the promoter was transfected into J558 plasmacytoma cells, the promoter displayed 3-fold higher activity than the

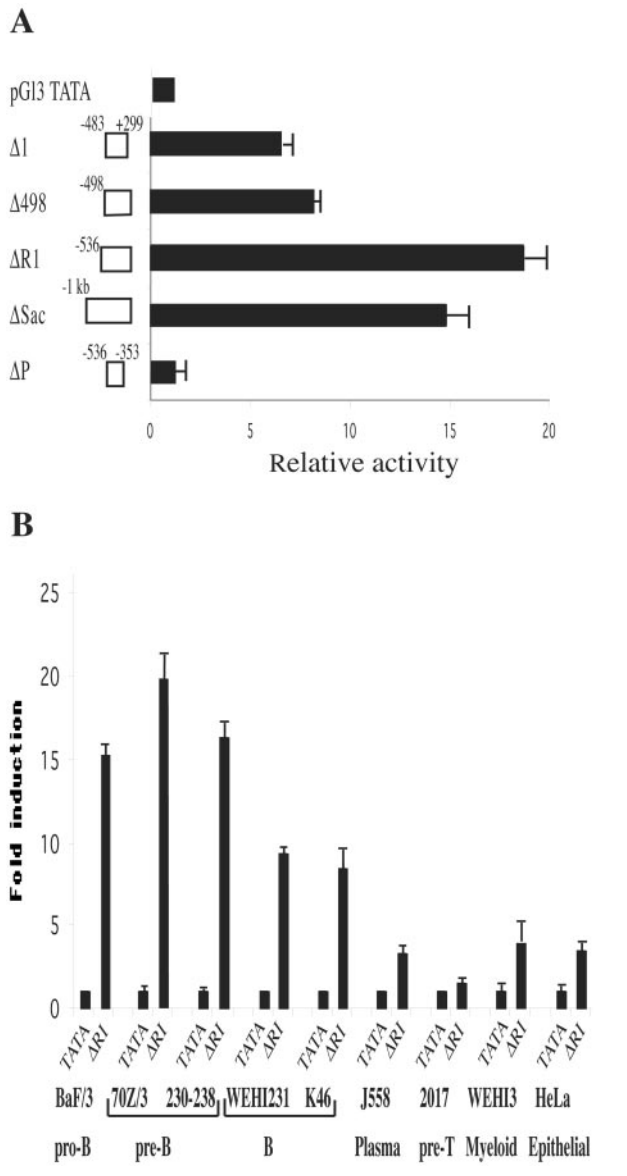

FIGURE 3. A tissue-restricted promoter is located 5′ of the EBF gene. *A*, A diagram with the resulting relative luciferase activity obtained after DEAE dextran-mediated transient transfections of 2 μg of the indicated *EBF* promoter reporter constructs into 70Z/3 pre-B cells. The nucleotide numbering refers to the promoter sequence in Fig. 2. *B*, A diagram with the resulting relative luciferase activity obtained after DEAE dextran-mediated transient transfections of 2 μg of the indicated reporter plasmids into a panel of cell lines. The reporter activity obtained with 2 μg TATA-box-controlled reporter plasmid was set to one. Data are collected from three transfections and the error bars indicate SD.

control plasmid while transfection into 2017 pre-T cells suggested that the promoter activities of the two constructs were essentially the same. The promoter was active to a low level in WEHI3 macrophage cells where the ΔRI construct was 3.5 times as active as the TATA-box reporter. Comparable 4-fold higher activity was also observed for the EBF promoter after transfection into the human epitheloid HeLa cell line. This suggests that the promoter displays a degree of restricted activity and therefore may directly participate in the regulation of stage and lineage specific transcription of the EBF gene.

The EBF promoter is a direct target for transactivation by EBF and E47

EBF has been suggested to be a genetic target for transactivation by the E2A protein E12 (25), or possibly the alternative splice form from the E2A gene, E47 (24). E47 and E12 both appear to bind to the same binding sites as homo- or heterodimers, even though E12 homodimers appear to do so with lower affinity than E47/E12 heterodimers or E47 homodimers (33). Both of these proteins are suggested to participate in a complex and essential network of transcriptional activators and repressors in the early B lymphocyte (37, 38). Thus, the presence of a potential binding site in the EBF promoter indicates that they may be involved directly in the regulation of the EBF gene. The ability of E2A proteins to interact with the E-box from the EBF promoter was examined in an EMSA using in vitro translated syrian hamster E47 (39) bound to the E2 E-box from the λ5 promoter (27) (Fig. 4*A*, *left panel*). The formation of this complex was not competed for by addition of the EBF site oligonucleotide, while the addition of EBF promoter E-box prevented the formation of the λ5E-box/E47 complex. The promoter also contained a potential binding site for EBF itself that would indicate that EBF might be involved in an autoregulatory loop. The ability of EBF to interact with its promoter was examined in an EMSA using recombinant in vitro-translated mouse EBF and the mb-1 promoter EBF binding site (14) (Fig. 4*A*; *right panel*). This indicated that while the EBF/mb-1 site complex was competed for by the addition of the potential EBF site, it remained unaffected by the addition of the EBF-promoter E-box containing oligonucleotide. These data suggest that both EBF and E47 have the ability to interact with the EBF promoter. To verify these findings, we used either the labeled EBF-promoter E-box or EBF site as probes and in vitro-translated E47 or EBF protein in additional EMSAs (Fig. 4*B*). None of the probes reacted to factors in the unprogrammed reticulocyte lysate while prominent complexes were detected using E47 programmed lysate and the E-box containing oligonucleotide from the EBF-promoter. The complex formation was reduced by the addition of the wild-type double-stranded E-box oligonucleotide but not by the inclusion of an oligonucleotide containing point mutations in the E-box. A similar result was found using rEBF and the EBF-promoter EBF site because a complex was formed that could be competed for by the inclusion of wild-type, but not point-mutated, EBF promoter oligonucleotides. These data further support the idea that both EBF and E47 have the ability to interact with the promoter element.

The binding of a transcription factor does not always result in increased functional activity of a promoter, so to investigate whether E47 and/or EBF were able to interact functionally with the promoter, we transiently transfected an EBF promoter reporter construct (Δ1) together with expression plasmids encoding EBF or E47 into epitheloid HeLa cells (Fig. 4*C*). Inclusion of 300 ng EBF expression plasmid resulted in a 2.3-fold induction of reporter activity while inclusion of 600 ng expression plasmid resulted in a 4.5-fold activity increase. Cotransfection of the EBF promoter with 300 or 600 ng E47 expression plasmid resulted in a 2- or 6-fold activity increase respectively. Transfection of a basal *fos*-promoter together with either EBF or E47 expression plasmids did not result in any significant induction of reporter activity. This indicates that the defined EBF promoter has the ability to functionally interact with both EBF and E47.

To investigate the binding of proteins to the EBF-promoter EBF site and E-box in nuclear extracts from a pre-B cell, we used the labeled EBF site and E-box and nuclear extracts from 70/Z3 cells in additional EMSAs (Fig. 5*A*). The EBF-promoter EBF site

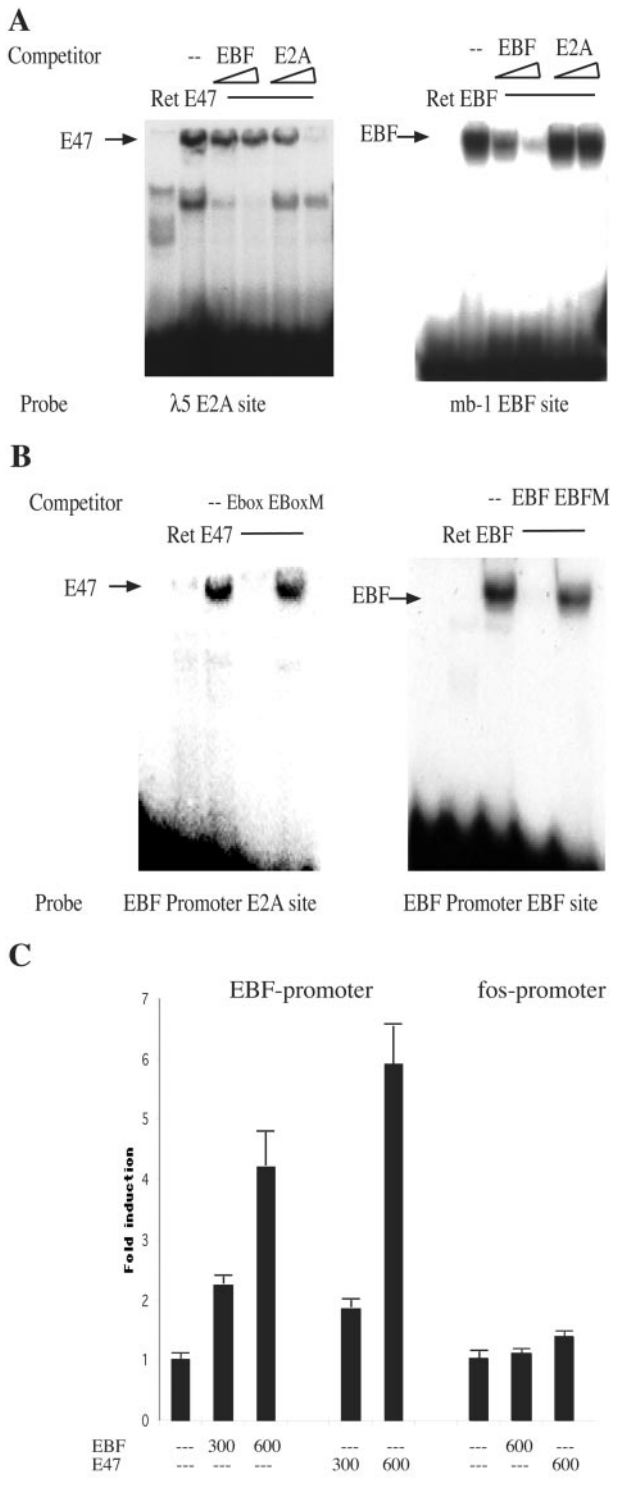

FIGURE 4. EBF and E47 are able to functionally interact with the EBF promoter. *A,* An EMSA analysis where the potential EBF and E2A binding sites are used to compete for binding of in vitro-translated E47 bound to the E2-Ebox from the λ*5* promoter (27) (*left panel*) or in vitro-translated EBF to the *mb-1* promoter EBF binding site (15) (*right panel*). The competitors are added in 150- and 500-fold excess. *B,* The resulting autoradiograms of EMSA experiments using rE47 or rEBF and labeled binding sites from the EBF promoter as indicated. The competitors were added in 500-fold excess. *C,* The resulting luciferase activity when EBF (Δ1) or cFos-promoter-controlled reporter constructs were transiently transfected into HeLa cells in combination with the indicated amounts of expression plasmids encoding EBF and/or E47 (27). The reporter activity obtained with 600 ng empty expression plasmid was set to one and data are collected from three transfections. Error bars indicate SD.

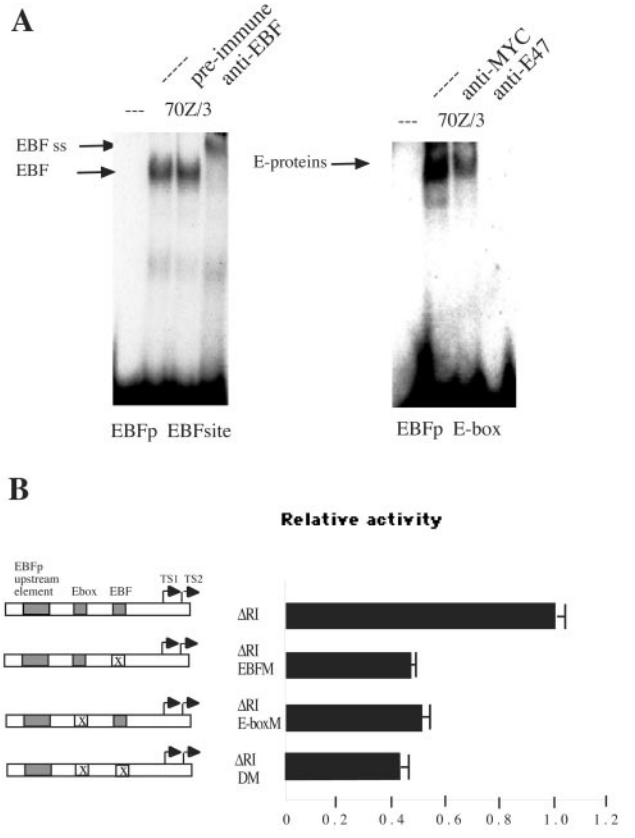

FIGURE 5. The EBF binding site and the E-box are important for the full functional activity of the EBF promoter in a pre-B cell line. *A,* EMSA supershift experiments where the EBF-promoter EBF site or E-box was incubated with nuclear extract from 70Z/3 cells in the presence of either preimmune sera, control Abs, EBF-antisera, or E47 Abs as indicated. *B,* The luciferase data obtained after transient transfection of 2 μg of either the wild-type ΔRI EBF promoter reporter or derivatives of this promoter carrying point mutations in either the EBF or E-box sites or both into 70Z/3 pre-B cells. The data are based on three transfections normalized to the expression of a cotransfected CMV *Renilla* plasmid and the activity of the ΔRI promoter was set to one. SD are indicated by error bars.

formed one major complex (EBF) that was nonreactive to preimmune sera while the complex was supershifted by the addition of anti-EBF anti-sera (R. Gisler and M. Sigvardsson, unpublished observations) suggesting that this site interacts with EBF in the complex protein mixture in a nuclear extract. The E-box spanning oligonucleotide formed one prominent complex with factors in the nuclear extract and this complex was only marginally affected by the inclusion of anti-*myc* Ab (9E10), while the addition of anti-E47 blocking Abs completely abrogated complex formation suggesting that the observed complex contains E2A proteins.

The importance of the E-box and the EBF site for promoter function in a pre-B cell line was then investigated by transient transfections of EBF promoters carrying point mutations in these sites into 70Z/3 cells (Fig. 5*B*). The activity obtained using an EBF site-mutated ΔRI construct was 48% that of the wild type, while the same promoter with a mutated E-box yielded 52% of this activity. Mutations of both the sites in the context of the ΔRI promoter resulted in a promoter with an activity comparable to that of the single mutants (43%). This suggests that both these elements are important for the full function of the promoter in a pre-B cell but that other elements still have the ability to stimulate transcription in the 70Z/3 cells.

Having data supporting the idea that EBF and E-proteins are involved in the regulation of the promoter in pre-B cells, we wanted to investigate potential differences in promoter activation in cells representing different stages of B cell development. To this end, we prepared nuclear extracts from Ba/F3 pro-B cells, 70/Z3 pre-B cells, WEHI231 B cells, and also WEHI3 macrophages. The integrity of the nuclear extracts was investigated by use of a decamer containing labeled oligonucleotide confirming that all the extracts contained intact Oct proteins (Fig. 6*A*). Using the EBF-promoter EBF site as a probe, we observed one major complex in the pre-B cells and a less prominent complex of comparable size in the B cells. The pro-B cell extract did not give rise to any prominent complex while the nuclear extracts from the macrophages contained a factor resulting in a prominent complex, but of an apparently different migration pattern, as compared with the complex in the pre-B and B cells (Fig. 6*A*). Two faint complexes were detected using nuclear extracts from the Ba/F3 pro-B cells and the EBF-promoter E-box, while an additional more prominent complex was detected in extracts from the pre-B and B cells (Fig. 6*A*). No prominent complex could be detected in extracts from the WEHI3 myeloid cells. These data suggested that the EBF site and E-box interacted differentially with nuclear factors from cell lines representing different stages of B cell development. To investigate the functional consequences of this, we transiently transfected the EBF site and E-box mutated EBF promoter reporter plasmids into Ba/F3, 70/Z3 and WEHI231 cells (Fig. 6*B*). Mutation in the EBF site resulted in a reduced function of the promoter in the 70/Z3 cells while this did not affect the function of the promoter in either the Ba/F3 pro-B or WEHI231 B cells. Mutation in the E-box resulted in a reduced function of the promoter in all the tested cell lines, but the effect was more pronounced in the 70/Z3 and WEHI231, than in the Ba/F3, cells. This indicates that the promoter may be regulated by different mechanisms at different stages of B cell development.

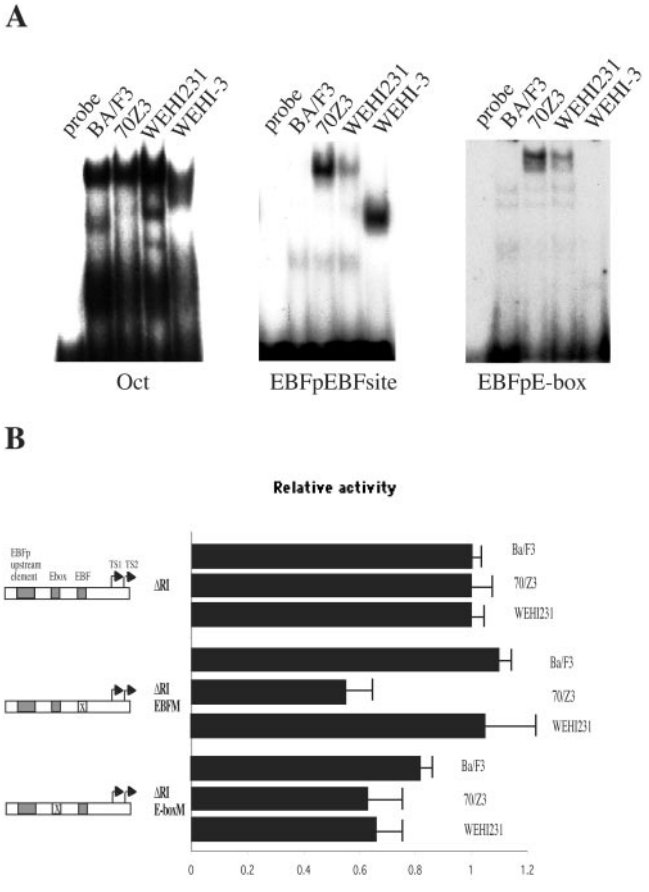

FIGURE 6. The EBF promoter is differentially regulated in cell lines representing different stages of B cell development. *A*, EMSA experiments where either a Oct protein binding site, EBF-promoter EBF site, or E-box spanning oligonucleotides has been incubated with 5 μg nuclear extracts from cell lines as indicated. *B*, The luciferase data obtained after transient transfection of 2 μg of either the wild-type ΔRI EBF-promoter reporter or derivatives of this promoter carrying point mutations in either the EBF or E-box sites into Ba/F3 pro-B, 70/Z3 pre-B, or WEHI231 B cells. The data are based on four transfections normalized to the expression of a cotransfected CMV *Renilla* plasmid and the activity of the ΔRI promoter was set to one. SD are indicated by error bars.

The 5′ part of the EBF promoter display restricted functional activity correlating with the binding of a nuclear factor

The mutation of the EBF site and the E-box together with transfection experiments in 70Z/3 cells suggested that the 5′ region between the Δ1 and the ΔRI (−536 to −483) contains a positive control element. To investigate the presence of nuclear factors with an ability to interact with this region, we made EMSAs with nuclear extracts from 70Z/3 pre-B cells and a labeled oligonucleotide spanning the 53-bp region between the Δ1 and ΔRI constructs (Fig. 7*A*). This resulted in several complexes displaying a rapid migration in the gel but also one apparently larger complex we denoted EBF promoter binding protein (EPBP). The formation of this complex could be competed efficiently for by the addition of a PCR-amplified promoter carrying the same region (ΔRI) while an amplified Δ1 promoter was largely inefficient in this respect. This indicates that the functionally active region possesses the ability to interact with nuclear factors in 70Z/3 pre-B cells. EMSA experiments using the downstream region as a probe and nuclear extracts from BaF/3 pro-B, 70Z/3 pre-B, WEHI231 B, and WEHI3 macrophage cells suggested that the protein only interacted with the DNA in extracts from WEHI231 and 70Z/3 cells, but not from Ba/F3 or WEHI3 cells (Fig. 7*B*). The integrity of the nuclear extracts was examined by an EMSA using an Oct protein binding consensus decamer. To further investigate the correlation between the function of the EBF promoter control element and protein binding, we made a series of transfections comparing the function of this element by transfections of the Δ1 and ΔRI reporter constructs in different cell lines (Fig. 7*C*). The element was a positive regulator in the 70Z/3 and WEHI231 cells while no apparent function of this element could be detected in Ba/F3 or WEHI3 cells suggesting that the function of the element correlates to binding and presence of EPBP.

To further define the binding site for EPBP, we made a set of EMSA competitions using point-mutated oligonucleotides spanning this control element. Mutations in the 3′ part (M1) of this element did not affect the ability of the resulting oligonucleotide to compete for complex formation between the 53-bp (−536 to −483) probe and factors in nuclear extracts from 70/Z3 cells (Fig. 8). In contrast, point mutations in the 5′ part of the element (M2–4) greatly impaired the ability of the corresponding double-stranded oligonucleotides to compete for complex formation indicating an essence for this part of the element in the interaction with EPBP. The region has been compared with known transcription factor binding sites both manually and in the Transfac database but without finding any homology to known binding sites. We have also performed supershift experiments using commercially available Abs against BSAP, *ets*1 and 2, Pu.1, Egr1, 2 and 3, NF-κB components P50, c-*rel* and *rel*B, E2A, and E2-2, but none of these was able to recognize the formed complex (data not shown).

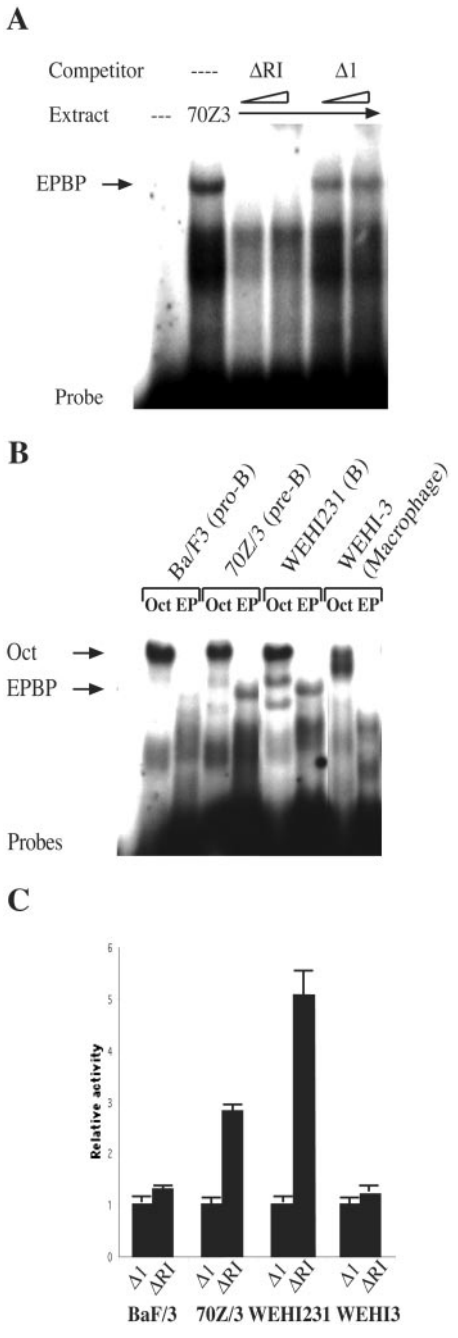

FIGURE 7. The 5′ region of the EBF promoter is mostly active and binds proteins in pre-B and B cell extracts. *A,* An EMSA gel where a labeled oligonucleotide spanning the region between −536 and −483 in the EBF promoter was incubated with nuclear extract from 70Z/3 cells. The obtained complex formation was competed for by the addition of unlabeled PCR-amplified EBF promoters spanning either the region −536 to −353 (ΔRI) or −483 to −353 (Δ1) as indicated. *B,* An EMSA where nuclear extracts from Ba/F3 pro-B-, 70Z/3 pre-B-, WEHI231 B-, and WEHI3 macrophage cell lines have been incubated with either an Oct binding decamer or the EBF promoter −536 to −483 oligonucleotide. *C,* A diagram of the relative functional activity of the −536 to −483 region after transient transfection or 2 μg of either Δ1 (set to one) or ΔRI into the indicated cell lines. Data are collected from three transfections and the error-bars indicate SD.

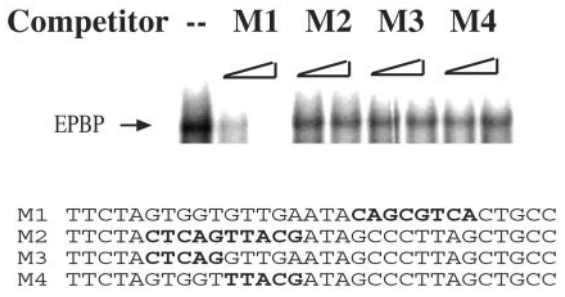

FIGURE 8. The EPBP complex is formed via interaction of a nuclear factor with a site located in the 5′ part of the defined EBF-promoter. EMSA competition experiments were the complex formation between the −536 to −483 region of the EBF promoter and protein nuclear extracts from 70/Z3 cells were competed for by the addition of point-mutated oligonucleotides as indicated. The full-length oligonucleotides are shown in *Materials and Methods*. The gel is cut to only display the EPBP complex.

Discussion

In this study, we report the identification of an E47 and EBF binding promoter region with tissue-restricted activity located 5′ of the mouse EBF gene. Even if the cloned EBF promoter displayed a more pronounced activity in cell lines with endogenous expression of the EBF gene, the defined element appears to be active upon transient transfections also in BaF/3 cells that have not fully entered the pro-B cell stage (16). This could indicate that this precursor cell already expresses factors capable to stimulate activation of the EBF promoter but that the promoter is inaccessible due to chromatin condensation or alternatively inactive due to CpG methylation of the transcription initiation sites. The involvement of promoter methylation in the regulation of the EBF gene is supported by the primary DNA sequence with a high GC content and the large number of CG combinations, allowing for cytosine methylation of the DNA (29, 30) (Fig. 2*A*). This is also supported by restriction enzyme analysis of the promoter, suggesting it to be resistant to digestion by the methylation sensitive enzyme *Hpa*II in BaF/3, but not 70Z/3 pre-B cells (E. M. K. Smith, R. Gisler, and M. Sigvardsson, unpublished observations). It is also most likely that other control elements are involved in the regulation of this gene and these may well differ among the varying cell types that express EBF. One possibly common feature of the regulation of the promoter in different cell types defined in this study comes from the finding that EBF may be involved in an autoregulatory loop. Positive feedback loops can also be found in other differentiation systems such as myogenesis where the basic-helix-loop-helix protein MyoD participates in its own regulation (40). The establishment of such loops may be of large importance for transcription factors involved in the definition and progression of a certain lineage.

The ability of the EBF promoter to directly interact with E47 is in line with the previous observation that ectopic expression of another E2A protein E12 resulted in expression of EBF in macrophage dedifferentiated 70Z/3 cells (25). This further establishes the suggested functional hierarchy of transcription factors in early B cell development where E2A proteins, and possibly the B cell restricted E47 homodimer (41–43), appear to play a key role in the absolute earliest stages of B cell development. E2A proteins regulate a number of early genes (44, 45) as well as recombination events (46) and EBF (25) expression. E47 and EBF also collaborate to activate a number of target genes and to promote B cell development in a synergistic manner (16, 19, 27). In addition, EBF appear to participate in the regulation of another set of genes, such as the B29 gene (17) and the BSAP-encoding Pax-5 gene (19). BSAP appear to collaborate with EBF in the activation of, for instance, the mb-1 (14, 47) and Blk genes (18), but also appear to act independent of EBF in the regulation of the *CD19* gene (48,

49). BSAP also appear to be crucial for the commitment of the B-lymphoid cell based on the finding that pro-B cells from BSAP-deficient, but not normal, mice, can be differentiated into a large number of hemopoietic lineages (50, 51). Thus, B cell commitment appears to involve a highly ordered sequence of molecular events where transactivating factors participate in the regulation of complementary genes as well as of each other. However, it should be noted that neither of these transcription factors are specifically expressed in B lineage cells suggesting that more has to be learned about transcription factor networks and epigenetics in early B cell development.

Our data suggest that EBF transcripts are initiated in the promoter region defined in this report, but it also becomes apparent that other control elements are crucial to obtain stage- and lineage-specific expression of the EBF gene. Thus, the cloning of this promoter element represents one step against the understanding of how the EBF gene is controlled and its continuation, including identification of EPBP and other regulatory elements, will further aid in the molecular elucidation of lineage initiation and commitment in the bone marrow.

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