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*J Immunol* 2002; 168:5130-5138; doi: 10.4049/jimmunol.168.10.5130

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The Human V-PreB Promoter Is a Target for Coordinated Activation by Early B Cell Factor and E47

Ramiro Gisler and Mikael Sigvardsson

The development of mature B lymphoid cells involves a highly orchestrated regulation of stage- and lineage-specific genes. In this study, we report an analysis of the human surrogate L chain VpreB promoter. The promoter has an overall homology of 56% to the mouse counterpart and displays a preB cell-restricted activity in transient transfections in cell lines. The promoter harbors three independent binding sites for early B cell factor (EBF) as defined by EMSA and supershift experiments. These sites were important for the full function of the promoter in a preB cell line, and chromatin immunoprecipitation experiments indicate that EBF interacts with the promoter in vivo. In addition to this, ectopic expression of EBF induces the activity of a reporter gene under control of the VpreB promoter in epithelioid HeLa cells, an effect augmented by coexpression of the basic-helix-loop-helix transcription factor E47. The ability to interact directly with E47 was shared by the promoters controlling the human mb-1 and B29 genes. These data indicate that the human VpreB promoter is a direct target for activation by EBF and E47 and that functional collaboration between these proteins may be of great importance in human B cell development. The Journal of Immunology, 2002, 168: 5130–5138.
Materials and Methods

5' end amplification of hVpreB cDNA

Amplification of the 5' end of the hVpreB gene was performed using the 5' RACE System for Rapid Amplification of cDNA Ends, version 2.0 (Life Technologies, Täby, Sweden). Five micrograms of total RNA from human Nalm6 preB cells was annealed to 100 nM first-strand primer (gene-specific primer (GSP1)) in diethylpyrocarbonate-treated H2O. The mixture, in a final volume of 15.5 µl, was incubated at 70°C for 10 min and chilled on ice, followed by the addition of 10× PCR buffer (20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl2; Life Technologies), 2.5 mM MgCl2, 10 mM dTT, and 400 µM dNTP mix. After incubation for 1 min at 42°C, 200 U SuperScript II RT polymerase (Life Technologies) was added, and synthesis of the first-strand cDNA was maintained for 50 min at 42°C. The reaction was terminated by heating the mixture at 70°C for 15 min. Degradation of the RNA strand in the RNA:cDNA complex was achieved by the addition of 1 µl RNase-free DNase I (Life Technologies) and incubation for 30 min. cDNA was purified using the GlassMAX Spin Cartridge (Life Technologies). Ten microliters of the purified cDNA was Dc-tailed by 30 cycles (94°C, 10 s; 55°C, 10 s; 72°C, 10 s), followed by a final extension step for 7 min at 72°C. The PCR product was amplified a second time with 2.5 U Taq-polymerase (Life Technologies) in the manufacturer’s buffer supplemented with 0.2 mM dNTP, in a total volume of 50 µl.

ESRase mix (Life Technologies) and antisense nested GSP were added to a final concentration of 400 nM. The Dc-capped 5' end of VpreB cDNA was amplified for 30 cycles (94°C for 30 s, 55°C for 30 s, and 72°C for 60 s), followed by a final extension step for 7 min at 72°C. The PCR product was amplified a second time with 2.5 U Taq-polymerase (Life Technologies) in the manufacturer’s buffer supplemented with 0.2 mM dNTP, in a total volume of 50 µl. The sense and antisense nested GSP were added to a final concentration of 200 nM. The template was amplified by 30 cycles (94°C for 30 s, 55°C for 30 s, and 72°C for 60 s). The product was then analyzed by sequencing using the Sequencing Kit (Applied Biosystems, Foster City, CA) with the sense oligo UAP and UAP (Life Technologies).

The oligonucleotides used for cDNA synthesis and amplification were as follows:

GSP1 sense, 5'-GCTTACACACCCGCAGTCTATG; GP2 antisense, 5'-GTCTCAGCAAGGCGGAGATG; and GP antisense, 5'-TGGTGACGGATACAAAAACAGATGG.


tissue culture conditions and cell lines

All cells were grown at 37°C in 5% CO2 in RPMI supplemented with 7.5% FCS, 10 nM HEPES, 2 mM pyruvate, 50 µM 2-ME, and 50 µg/ml gentamicin (all purchased from Life Technologies). The medium for the Ba/F3 cells was supplemented with 10% conditioned medium from confluent WEHI3 cells as a source of IL-3. BaF3, WEHI3, and HeLa cells were gifts from Dr. R. Grosschedl, the 18-81 and 230B8 Abelson transformed pre-B cell lines (originally defined by Dr. Rosenberg et al.) were gifts from Dr. T. Leander, MlB2 cells were gifts from Dr. J. Hagman, and Namalwa B cells as well as Jurkat T cells were gifts from Dr. C. Broker.

transient transfections and luciferase assays

Five hundred thousand cells were washed once with serum-free medium (OptiMEM, Life Technologies) and taken up in 800 µl medium for transfection. Lipofectin (5 µl; Life Technologies) and a 5 fold molar excess of the mixing oligonucleotides and antisense GSP were added to a final concentration of the mixture at 37°C for 30 min. cDNA was purified using the GlassMAX Spin Cartridge (Life Technologies). Preparation of protein extracts and luciferase assays were performed with a Dual-Luciferase Reporter Assay System (Promega) using 20% of the total protein extract. The obtained luciferase activity was normalized against the activity of a cotransfected (0.25 µg) CMV-controlled Renilla luciferase reporter gene.

Protein extracts and EMSA

Nuclear extracts were prepared according to the method reported by Schreiber et al. (26) DNA probes were labeled with [γ-32P]ATP by incubation with T4 polynucleotide kinase (Roche, Mannheim, Germany), annealed with the complementary strand, and purified on a 5% polyacrylamide tris-borate-EDTA gel. Five to 10 µg nuclear extract or 0.5–2 µl in vitro-transcribed/translated protein was incubated with the labeled probe (20,000 cpm, 3 ml) for 30 min at room temperature in binding buffer (10 mM HEPES (pH 7.9), 70 mM KCl, 1.0 mM DTT, 1.0 mM EDTA, 2.5 mM MgCl2, and 0.05% Nonidet P-40) with 0.75 µg poly(dIdC) (Pharmacia, Uppsala, Sweden). ZnCl2 (1 mM) was added to shift assays performed in the presence of EB. DNA competitors were added 10 min before addition of the DNA probe. The samples were separated on a 6% acrylamide tris-borate-EDTA gel, which was dried and subjected to autoradiography. Competitors based on synthetic oligonucleotides were added to the molar excesses indicated in the respective figures. Full-length mb-1, B29, and VpreB promoters were generated by PCR (see below) and were added at the molar excesses indicated in the respective figures. Supershifts were performed under the same conditions, but with the additional presence of 2 µl polyclonal EB Ab (Innovagen, Lund, Sweden) or 2 µl primary serum (Innovagen) as a negative control.

The oligonucleotides used for EMSAs were (underlined core sequences indicate the nucleotides comprising the core site): mb-1 sense, 5'-AGCCACCTCCCTCCAGGTTTGTGG; mb-1 antisense, 5'-CCACATTTTCCTAGAGGAAGCTGTAGCTACACTTGTTGCT; mutated mb-1 sense, 5'-GGCCAGAAGCTCTCCAGGACG; mb-1 antisense, 5'-CGTCTGAGGCTTCCTGTGGC; oct binding site sense, 5'-CATCCTCAAGTTGATGGTCCATGAGACCTAGCTG; and antisense, 5'-CATCTGCTATGGATGCTAGACACAGTTGAGCCAGAGCGCC.

The oligonucleotides used for EMSAs and amplification were as follows: GSP1 antisense, 5'-GCTTACACACCCGCAGTCTATG; GP2 antisense, 5'-GTCTCAGCAAGGCGGAGATG; and GP antisense, 5'-TGGTGACGGATACAAAAACAGATGG.


tissue culture conditions and cell lines

All cells were grown at 37°C in 5% CO2 in RPMI supplemented with 7.5% FCS, 10 nM HEPES, 2 mM pyruvate, 50 µM 2-ME, and 50 µg/ml gentamicin (all purchased from Life Technologies). The medium for the Ba/F3 cells was supplemented with 10% conditioned medium from confluent WEHI3 cells as a source of IL-3. BaF3, WEHI3, and HeLa cells were gifts from Dr. R. Grosschedl, the 18-81 and 230B8 Abelson transformed pre-B cell lines (originally defined by Dr. Rosenberg et al.) were gifts from Dr. T. Leander, MlB2 cells were gifts from Dr. J. Hagman, and Namalwa B cells as well as Jurkat T cells were gifts from Dr. C. Broker.

transient transfections and luciferase assays

Five hundred thousand cells were washed once with serum-free medium (OptiMEM, Life Technologies) and taken up in 800 µl medium for transfection. Lipofectin (5 µl; Life Technologies) was diluted in 100 µl serum-free medium, incubated for 45 min at room temperature, and then 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 CO2 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 4 h, and protein extracts were prepared directly in the 24-well plate by adding 80 µl cell lysis buffer (Promega, Falkenberg, Sweden). The luciferase assay was conducted using 20 µl of the obtained extracts and 200 µl luciferase assay reagent (Promega). Preparation of protein extracts and luciferase assays were performed with a Dual-Luciferase Reporter Assay System (Promega) using 20% of the total protein extract. The obtained luciferase activity was normalized against the activity of a cotransfected (0.25 µg) CMV-controlled Renilla luciferase reporter gene.

Plasmids and constructs

The human EBV expression plasmid was based on the eukaryotic expression vector pcDNA3 (Invitrogen, Leek, The Netherlands), which places the inserted human EBV cDNA under the control of a CMV promoter (25). The human mb-1 (284 to translation start-2), B29 (146 to +54) (25), and VpreB (270 to +27) promoters were PCR-amplified using promoter-specific sense and antisense primers with genomic HeLa cDNA as template, resulting PCR products were cloned in the Smal site of the luciferase reporter plasmid plG3 Basic (Promega). The VpreBshort M (293 to +27) and VpreBshort (293 to +27) promoters were amplified using the VpreB (246 to +27) promoter construct as a template. The point mutations in the VpreBshort M sequence were introduced using mutated sense and antisense primers. Both short constructs were cloned in the Smal site as described above. All constructs were verified by sequencing.

The oligonucleotides used for PCR of the B29 promoter construct were sense, 5’-TGGTGACGGATACAAAAACAGATGG; antisense, 5’-GTGACGAGCCAGCCCTTGAACCA; mb-1 PCR antisense, 5’-CTCTCCACGTAGCTACACTTGTTG; B29 PCR sense, 5’-CCTCGGTACCCTTCTCAGG; B29 PCR antisense, 5’-GACCTCGTACCCTTCTCAGG.
CCCTAAGTGACCTTAACCCAAAGGCCTCCAATGCACTGGCCCCAGAGTCTCC; and mutated VpreBshort PCR antisense, 5’-CATGGTCA GACATGCGAGCTGAGTC TCTGTGGCCACGGGCTGGGACGT GCCCTGCTCATTAGCAAATGGCAGCATCCTCCTCCTG.

In vitro transcription and translation
Recombinant protein was generated by coupled in vitro transcription/translation using a reticulocyte lysate kit (Promega).

Chromatin immunoprecipitation assay
Detection of in vivo interaction between the VpreB promoter and EBF was performed using the chromatin immunoprecipitation (ChIP) assay kit (Upstate Biotechnology, Lake Placid, NY). Briefly, the proteins of 1–4 × 10⁶ Nalm6 HOK preB cells were cross-linked to the genomic DNA by addition of 100 μl 37% formaldehyde. The treated cells were incubated for 10 min at 37°C, washed twice with ice-cold PBS containing protease inhibitors, and pelleted. The pellet was resuspended by the addition of 200 μl SDS lysis buffer (Upstate Biotechnology) and incubated on ice. The ice-cold lysate was sonicated and pelleted by centrifugation. The supernatant, containing the sonicated DNA, was diluted by the addition of ChIP dilution buffer (Upstate Biotechnology) and salmon sperm DNA/protein A agarose-50% slurry (Upstate Biotechnology) and incubated with rotation for 30 min at 4°C. The mixture was centrifuged, and the supernatant was incubated overnight at 4°C with rotation together with 60 μl salmon sperm DNA/protein A agarose-50% slurry and 2 μl polyclonal EBF Ab, followed by the

FIGURE 1. The 5’ region of the human VpreB gene contains a stage- and lineage-specific promoter. A, Sequence alignment of the human and the mouse VpreB promoters. The 5’ end obtained by RACE analysis of the human promoter is indicated by a black dot, while potential transcription factor binding sites are indicated by alignment to their consensus sequences. The stars represent the nucleotides in the mouse promoter that match the human counterpart, while the dashes represent mismatches due to deletions or insertions. The arrows represent transcriptional start sites in the mouse promoter (48).

B, Relative luciferase activities obtained after transient transfections of either a basal promoter or the human VpreB promoter (~426 to +27) into a set of cell lines as indicated. The transfection results were normalized against a cotransfected CMV Renilla luciferase plasmid, and the data represent three transfections. The error bars indicate SDs.
addition of 60 μl salmon sperm DNA/protein A agarose-50% slurry and incubation at 4°C for 1 h. A negative control experiment using 2 μl pre-immune serum was performed simultaneously. The agarose/Ab/histone complex was washed with the manufacturer’s washing buffer and eluted with 2×250 μl elution buffer (1% SDS and 0.1 M NaHCO3). The histone-DNA cross-links from the combined eluates were reverted by addition of 20 μl of 5 M NaCl and incubation at 65°C for 4 h. Following this, 10 μl of 0.5 M EDTA (Upstate Biotechnology), 20 μl of 1 M Tris-HCl (pH 6.5; Upstate Biotechnology), and 2 μl of 10 mg/ml protease K were added to the mixture and incubated at 45°C for 1 h. The DNA was recovered by extraction with phenol/chloroform, followed by ethanol precipitation and the addition of 20 μg glycogen. Genomic VpreB DNA was subsequently amplified from this sample in a PCR with 5 U Taq polymerase (Life Technologies) in the manufacturer’s buffer supplemented with 0.2 mM dNTP in a total volume of 100 μl. The sense and antisense primers were added to a final concentration of 1 μM. As a negative control a PCR was performed using Rag-1 primers, followed by an additional PCR using a nested Rag-1 antisense primer. The PCR products were blotted onto Hybond N+ nylon membranes (Amersham) 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 55°C for 90 min and hybridized with 32P-labeled oligonucleotide for 12 h at 55°C in the same solution. Membranes were washed twice in 2× SSC for 15 min each time and once in 0.1× SSC/0.1% SDS for 15 min at room temperature. The DNA was disrupted in fragments ranging in size from 200 to 1000 bp by sonication (Vibra Cell Processor with eight sets of five pulses, with an amplitude of 10 and a 3-mm tip).

The oligonucleotides used for PCR were: VpreB sense, 5′-CT GGGCTGCTGTCTGCTTCAGGC; VpreB antisense, 5′-GCTGTACA CACCGGAGTGCT; and VpreB hybridization, 5′-TGT GCAGTACGCAAACAGGATGG (GSP antisense). The oligonucleotides used for PCR were: mb-1 sense, 5′-TTGCCCTCTTGTT TCTCAGAACAT; and mb-1 antisense, 5′-TTGTCCCTTGTT TCTCAGAACAT; and mb-1 nested antisense, 5′-CTTAGCTCAG GGGGCGCTTGAGT.

Results

The human VpreB gene is located in the Igα locus on chromosome 22q band 11.2, but a detailed characterization of a promoter element has not been reported. Thus, to identify the 5′ end of the mRNA, we performed RACE using RNA from the human preB cell line Nalm6 and primers located in exon 2 of the VpreB gene. This resulted in one distinct PCR product of ~120 bp, and products from two independent experiments were sequenced by a nested primer. Both these products resulted in exactly the same sequence, which was aligned to genomic DNA in a GenBank Blast search. The homology to the genomic clones was interrupted by the C tail added to allow for PCR amplification 27 bp upstream of the translation initiation codon (Fig. 1A). The overall homology of this putative promoter region to the mouse VpreB1 promoter was 56%, and using the Transfac database for analysis of the 5′ sequence suggested several potential binding sites for transcription factors, including STAT, IKAROS, c-Myb, GATA proteins, LMO2, EBF, and basic helix-loop-helix proteins (E boxes) (27).

We were unable to define a TATA box, but the region surrounding the 5′ end of the cDNA defined by RACE contained several potential initiator core sequences (28).

To investigate whether the region 5′ of the human VpreB gene was able to function as a promoter element we PCR-amplified a 453-bp fragment spanning the region from −426 to +27 and cloned the potential promoter fragment in front of a luciferase reporter gene. The plasmid was transiently transfected into a series of cell lines representing different lineages or developmental stages of the B lymphocyte. To obtain a relative approximation of functional activity within the different

\[ \text{FIGURE 2. The VpreB promoter contains functional binding sites for EBF. A, Autoradiogram showing EMSAs where the binding of in vitro-translated recombinant human EBF to the human mb-1 promoter EBF binding site was competed for by the addition of duplex oligonucleotides spanning the potential EBF binding sites in the VpreB promoter. B, Resulting luciferase activity when VpreB (−426 to +27) or c-fos promoter-controlled reporter constructs were transiently transfected into HeLa cells in combination with the indicated amounts of expression plasmids encoding human EBF. The DNA content in the transfections was normalized by the addition of the empty expression vector. The reporter activity obtained with 600 ng empty expression plasmid was set at 1, and data were collected from three transfections.} \]
cell types, we compared the activity obtained with the VpreB promoter to that obtained with a strong TATA box initiator element without any upstream regulatory binding sites (29) (Fig. 1B). Transfection of the VpreB promoter reporter into VpreB-negative mouse BA/F3 pro-B cells resulted in a relative activity of 0.5, while transfection of the mouse preB cell lines 18-81 and 230/238 resulted in 6- and 3-fold relative activities. We also transfected human preB cell lines with a large number of different protocols (data not shown), but low overall transfection efficiency made us rely on mouse cell lines for these specific experiments. Transfection into mouse (M12) and human (Namalwa) mature B cells resulted in relative activities of 0.3 and 0.1, while the activities obtained in human Jurkat T cells and epithelioid HeLa cells resulted in 0.3 and 0.4 times the activity of the control promoter. Thus, the human VpreB promoter displays a higher relative activity in preB cells compared with cells at other stages or from other lineages. These data suggest that the human VpreB gene is flanked by a stage- and lineage-restricted promoter.

The human VpreB promoter is a target for activation by EBF

In the mouse, the VpreB gene appears to be a direct target for the activity of the transcription factor EBF. This protein interacts directly with the promoter (30), and ectopic expression of EBF induces the endogenous gene in BA/F3 cells (20). The mouse promoter contains one high affinity binding site for EBF important for full functional activity of the promoter (30). Investigation of the nucleotide sequence of the human promoter revealed several potential EBF binding sites with varying degrees of similarity to the defined EBF consensus site ATTCCTNGGGAAT (31) (Fig. 1A). To investigate whether any of these sites was able to interact with EBF in vitro, we performed EMSA. In vitro-translated human EBF was bound to the mb-1 promoter EBF site. The complex formation was competed for by the addition of unlabeled duplex oligonucleotides spanning the potential EBF sites from the VpreB promoter to allow for the identification of functional binding sites (Fig. 2A). A duplex oligonucleotide encompassing EBF site 1, with nine bases matching the consensus site, was unable to compete for complex formation, while site 2, with nine matching bases, possessed this ability. Site 3, corresponding to the major binding site in the mouse VpreB promoter (30) with seven bases matching the consensus, was unable to compete efficiently. The same was found for site 4, while site 5, with eight matching bases, was able to compete for complex formation. Sites 6 and 8 could not compete efficiently, while site 7, containing nine matching bases, had this ability. Thus, the human EBF promoter contains at least three independent sites able to interact with EBF in vitro.

FIGURE 3. Mutations in the defined VpreB promoter EBF sites disrupt binding and functional activation of the VpreB promoter. A, Schematic drawing of the deletion and the mutations introduced into the VpreB promoter to inhibit the binding of EBF. The shaded nucleotides represent the point mismatches introduced in the mutated EBF binding sites. B, EMSA analysis where binding of in vitro-translated hEBF was competed for by the addition of PCR-amplified VpreB promoters as indicated. C, Resulting luciferase activity when the truncated (−293 to +27) or point-mutated (−293 to +27) VpreB promoter- or c-fos promoter-controlled reporter constructs were transiently transfected into HeLa cells in combination with 600 ng of either empty or hEBF-encoding expression plasmid. The reporter activity obtained with 600 ng empty expression plasmid was set at 1, and data were collected from three transfections. Error bars indicate the SDs.
To investigate whether the binding of hEBF to the VpreB promoter resulted in functional activation, we transfected the VpreB promoter reporter plasmid into epithelioid HeLa cells together with increasing amounts of hEBF encoding expression plasmid (Fig. 2B). The activity of the reporter gene increased from 2- to 3-fold to 8-fold with increasing amounts of EBF, while no effect was observed for a basal Fos promoter, suggesting that EBF interacts functionally with the VpreB promoter.

To verify the identity of the binding sites and to clarify that the direct binding of hEBF to these sites is necessary for hEBF-mediated activation, we constructed a VpreB promoter with point mutations in the defined EBF sites. To create such a promoter we made a deletion resulting in a DNA fragment extending from EBF binding site 2 to the translational start site (−293 to +27, VpreB-short; Fig. 3A). This deletes one of the E boxes while the defined EBF binding sites are kept intact. The EBF sites were then mutated (VpreBshort M) by PCR using oligonucleotides carrying point mutations at sites 2, 5, and 7 (Fig. 3A). Truncated as well as point-mutated promoters were then amplified by PCR and used as competitors in an EMSA as described above (Fig. 3B). The truncated promoter competed efficiently for complex formation, while the point-mutated promoter did not, suggesting that the introduced mutations severely impaired EBF binding. To investigate the ability of EBF to activate the VpreB promoter, we cloned the short wild-type as well as point-mutated promoters in the luciferase reporter vector and transfected the obtained constructs into epithelioid HeLa cells either alone or together with an EBF expression plasmid (Fig. 3C). The activities of all the promoters were comparable when transfected together with empty expression plasmid. Inclusion of the EBF expression vector induced the truncated promoter 6-fold, while the mutated as well as a control basal Fos promoter were essentially unaffected by this. This confirms that EBF can bind to and specifically activate the human VpreB promoter in a nonlymphoid cell line.

EBF is involved in regulation of the VpreB promoter in preB cells

As EBF was able to functionally interact with the VpreB promoter we wanted to investigate whether EBF participated in the regulation of the promoter in a preB cell. To this end we raised a rabbit

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**FIGURE 4.** EBF interacts with functionally important sites in the VpreB promoter in preB cells. A, EMSA where recombinant mouse as well as cellular human EBF in complex with a mb-1 promoter EBF site are incubated with preimmune serum or an antiserum raised against a region in the carboxyl-terminal part of EBF as indicated. The EBF complex is indicated by an arrow. The autoradiograms in B show the results when the human VpreB promoter EBF sites were incubated with nuclear extracts from human Nalm6 preB cells and preimmune or EBF antiserum. The right panel displays the results when a decamer-containing oligonucleotide was incubated with Nalm6 nuclear extract and the antiserum as described above. C, Southern blot analysis of the PCR products obtained after immunoprecipitation of formaldehyde-cross-linked human Nalm6 chromatin DNA. Lane 1, ChIP assay performed with the addition of hEBF antiserum; lane 2, ChIP assay performed with preimmune non-EBF-reactive antiserum; lanes 3 and 4, PCR controls using purified HeLa cell DNA as the positive control and distilled water as the negative control. D, Relative luciferase activities obtained after transient transfections of either the wild type (−426 to +27), truncated VpreBshort (−293 to +27), or EBF point-mutated (VpreBshort M) human VpreB promoter into 18-81 mouse preB cells as indicated. The activity obtained with the wild-type promoter was set at 1. The transfection results were normalized against a cotransfected CMV Renilla luciferase plasmid, and the data represent three transfections. The error bars indicate SDs.
antiserum against a conserved region in the carboxyl terminus of human and mouse EBF. The function and specificity of the antisera were verified by supershift using recombinant mouse or Nalm6 nuclear extract hEBF bound to the mb-1 promoter EBF site (Fig. 4A). No effect was seen when preimmune serum was added, while inclusion of immune serum resulted in a prominent supershift of the complex. This suggests that the antiserum is able to interact with hEBF in an EMSA and therefore can be used to identify EBF/DNA complexes in nuclear extracts. The ability of the VpreB promoter EBF sites to bind EBF in a nuclear extract was verified in EMSAs using labeled binding sites and nuclear extracts from the human preB cell line Nalm6 (Fig. 4B). All three defined binding sites were able to interact with a factor reactive with the EBF antiserum, suggesting that they interact with EBF in a nuclear extract from a human preB cell. The complex obtained with a decamer-containing, Oct-binding probe was not affected by addition of the antiserum, suggesting that the supershifting ability of the antiserum was specific. To further investigate the interaction of EBF with the VpreB promoter, we used the EBF antiserum in a ChIP experiment. Although the preimmune serum was unable to precipitate by PCR any detectable amount of the VpreB promoter DNA, the anti-EBF serum precipitated material sufficient to be detected after 30 cycles of PCR by hybridization of the blotted PCR product to a 32P-labeled internal oligonucleotide (Fig. 4C). As an additional control, none of the antiserum was able to precipitate material for amplification of the human Rag promoter from the same cells (data not shown). This indicates that EBF binds to the VpreB promoter in a preB cell in vivo. Knowing that EBF has the ability to interact with the VpreB promoter we aimed to investigate whether the EBF binding sites were important for promoter function in a preB cell line. To this end we transfected the mouse preB cell line 18-81 with the wild-type as well as the mutated VpreB promoter plasmids (Fig. 4D). The activity of the wild-type (−426 to +27) promoter is set at 1, and the shorter promoter lacking one E box (−293 to +27) displayed 45% of the wild-type activity, while the point-mutated (−293 to +27) promoter retained 9% the activity of the wild-type promoter. These data indicate that EBF binds to and participates in regulation of the VpreB promoter in a preB cell.

**EBF and E47 share target genes in human B cell development**

In addition to being a direct target for EBF, the mouse VpreB promoter is also a target for the basic helix-loop-helix protein E47, which has been shown to act in synergy with EBF in the induction of the promoter (20) (M. Sigvardsson, unpublished observation). A synergy between EBF and E47 can also be seen on the mouse A5 (20) and mb-1 promoters (M. Sigvardsson, unpublished observation). Thus, it appears that the coordinated activity of E47 and EBF is important in the developing mouse B lymphocyte. We have presented data suggesting that the promoter of the human A5 homologue 14.1 contains binding sites for both EBF and E47 and that these proteins can cooperate to activate the promoter (25). To expand our observation we decided to investigate the ability of the human B29, mb-1, and VpreB promoters to interact with E47. To this end we incubated in vitro-translated E47 with a labeled oligonucleotide spanning the µE2 site from the murine IgH intron enhancer (20). Formation of the DNA protein complex was then competed for by the addition of PCR-amplified promoter regions (Fig. 5A). Although the B29, mb-1, and VpreB promoters were able to compete for complex formation, the CD19 promoter (32) was not, suggesting a specific interaction between the first three promoters and E47. To further identify the binding sites for E47 in the VpreB promoter, we made a second set of competition experiments using oligonucleotides spanning the three E boxes in the VpreB promoter (Fig. 5B). All three were able to compete for binding of recombinant E47 to the µE5 E box, suggesting that this promoter contains at least three independent binding for E proteins. To investigate whether the binding of both EBF and E47 to these promoters resulted in a functional cooperation, we transfected reporter constructs into epithelioid HeLa cells together with empty expression vector, EBF, and/or E47 expression vectors. The inclusion of 300 ng EBF expression plasmid resulted in a 2.5-fold induction of the VpreB promoter (20) and the synergy between EBF and E47 can also be seen on the mouse VpreB promoter (20), and the synergy between EBF and E47 can also be seen on the mouse VpreB promoter (20).
the VpreB promoter, while inclusion of E47 plasmid did not significantly affect the transcriptional activity of the promoter (Fig. 5C). The combination of EBF and E47 expression plasmids resulted in a 16-fold induction of reporter activity, while no effect was seen on the basal Fos promoter that was used as a control construct (Fig. 5B). This indicates that EBF and E47 have the ability to act in synergy on the human VpreB promoter. Collaboration, although not as dramatic as on the VpreB promoter, could also be seen on the B29 and mb-1 promoters in the same experimental system (data not shown). Thus, we suggest that EBF and E47 collaborate in the induction of promoters regulating genes of importance for definition of the human preB cell.

Discussion

In this study, we present data suggesting that the human VpreB promoter is a direct target for trans-activation by EBF and E47 and that the collaborative action of these proteins can be seen on several promoters participating in the control of genes in human preB cells. The mb-1 and B29 promoters display ~70–75% sequence homology between man and mouse (33, 34). Even though several of the known transcription factor binding sites, including those for EBF, carry nucleotide differences, there is an apparent conservation of the functionality of these sites between species (33, 34). The same is true for the E protein E box motifs (CAGG/CTG) (35) that are conserved, while another type of E box in the mb-1 promoter is lost in humans (33). The human B29 promoter, in contrast, carries an extra E box compared with the mouse counterpart (34), but the overall anatomies of the promoters appear to be conserved. The situation in the 14.1 promoter is strikingly different, because the overall homology is ~50% (36), with none of the EBF sites and only one of the E boxes conserved (37). Instead, this promoter carries five EBF sites and two E boxes that cannot be found in the mouse counterpart (25), suggesting a conserved functionality extending beyond conservation of the promoter structure. The VpreB promoter has an overall mouse to human homology of 56%, and one EBF site as well as one E box appear to be conserved. However, the human promoter contains at least two more EBF sites and two more E boxes, while one mouse EBF site is lost, features that may extensively alter the promoter anatomy. The location of the human VpreB1 gene in relation to the 14.1 gene is also different from that in the mouse counterpart (38), because the mouse VpreB1 and λ5 genes are located only ~5 kb from each other, possibly allowing for direct coregulation (13, 14). The human VpreB1 and 14.1 genes are both located in the λ locus on chromosome 22, but at a rather large distance from each other (38, 39), a feature complicating coregulation of these genes in cis.

The finding that EBF also interacts with both the human Blk (R. Gisler, unpublished observation) and 14.1 (25) promoters suggests that this factor is a key coordinator of the expression of genes involved in the assembly of the preB cell receptor. The need for such a factor is supported by the fact that all components of the receptor appear to be nonredundant, and the loss of any of these proteins results in disturbances of B cell development in mice (15, 16, 40, 41). The genes encoding these crucial proteins are dispersed in the genome, with the mb-1 gene on human chromosome 19, and the B29, 14.1, and VpreB genes in different positions on chromosome 22. This complicates regulated coexpression due to a common locus control region or other cis-acting elements and introduces the need for coordination via trans-acting factors or possibly colocalization of chromatin to a certain region in the nucleus. The model that can be suggested from our experiments is that the combination of EBF and E47 act to coordinate the transcription of the genes providing the protein components of the preB cell receptor. Another aspect in need of consideration is the differences in expression patterns of, on the one hand, the surrogate L chain and, in contrast, the B29 and mb-1 genes (10, 42). This has been attributed to active repression of the mouse λ5 gene (43), but may also be a result of the fact that the expression of EBF is downregulated in mature B cells (25, 44). This would result in a promoter activity drop that potentially would also reduce transcription from the mb-1 and B29 genes. Such an effect could be prevented by a transcription factor relay race, where EBF and E47 act on all four genes in the pro-B cell, while other factors are the main-activators of the mb-1 and B29 promoters at a later stage of development. This model is supported by studies of the mouse B29 (45, 46) and mb-1 (47) promoters, where the relevance of different binding sites for promoter function varies with the differentiation stage of the B cell. A relay race model is also interesting in the context of lineage-specific gene activation, because epigenetic changes might be introduced by the factors that initiate transcription from a silent gene. These changes could then be inherited by factors acting on a control element at a later stage of development, abolishing the need for these secondary factors to be capable of remodeling chromatin.

A striking feature emerging from this study is the apparent conservation of genetic networks that appear to extend beyond conservation of the primary DNA sequence of promoter elements. This indicates that both EBF and E proteins are of great importance for human B cell development, motivating further efforts to elucidate their role and function in normal as well as malignant B cell development.

Acknowledgments

We thank Dr. Susanna Cardell and Jens Wrammert for helpful comments and critical reading of the manuscript, and Dr. Steffen Junker for cell lines.

References


