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Early B Cell Factor Is an Activator of the B Lymphoid Kinase Promoter in Early B Cell Development

Peter Åkerblad and Mikael Sigvardsson

Early B cell factor (EBF) is a transcription factor suggested to be involved in the transcriptional control of several B cell restricted genes. EBF is also essential for B lymphocyte development because mice carrying a homologous disruption of the EBF gene lack mature B lymphocytes. This makes the identification of genetic targets for EBF important for the understanding of early B cell development. Examination of the nucleotide sequence of the B lymphoid kinase (Blk) promoter suggested the presence of an EBF binding site, and in vivo footprinting analysis showed that the site was protected from methylation in a pre-B cell line. EMSA indicated that recombinant and cellular EBF interact physically with this site; furthermore, transient transfections indicated that ectopic expression of EBF in nonlymphoid HeLa cells activate a Blk promoter-controlled reporter construct 9-fold. The defined EBF binding site was also important for the function of the Blk promoter in pre-B cells, because transient transfections of a reporter construct under the control of an EBF site-mutated Blk promoter displayed only 20–30% of the activity of the wild-type promoter. Furthermore, transient transfections in HeLa cells proposed that EBF and B cell-specific activator protein were able to cooperate in the activation of a Blk promoter-controlled reporter construct. These data indicate that EBF plays an important role in the regulation of the Blk promoter in early B cell development and that EBF and BSAP are capable to act in cooperation to induce a target gene. The Journal of Immunology, 1999, 163: 5453–5461.

The development of B lymphocytes involves a complicated differentiation pathway, demanding a correct expression of stage and lineage specific genes (1–3). This is achieved by the interaction of transcription factors with promoter and enhancer elements. The importance of distinct transcription factors during B cell differentiation has been shown from experiments where disruption of genes encoding certain transactivators results in impaired B cell development (4, 5). One transcription factor shown essential for B cell development is early B cell factor (EBF) (6). Mice carrying a homologous disruption of the EBF-encoding gene display a B cell differentiation block at the pro-B cell stage (7, 8), before initiation of Ig heavy chain (IgH) recombination (9). EBF is expressed at all stages of B cell development except in the plasma cell stage, and also in neuronal cells and adipose tissue (6, 10). The protein interacts as a homodimer with the DNA core sequence CCCNNGGG (11) by a DNA binding domain containing a zinc-coordination motif (12). EBF has been shown to interact with functionally important regions in the promoters of mb-1 (13), B29 (14), Λ5 (15–17), and VpreB (15, 18). These genes encode the Ig-associated α- and β-chains (19, 20) of the pre-B and B cell receptors (pre-BCR, BCR) and the surrogate light chains of the pre-BCR (21, 22). This suggests that EBF has a role in the regulation of several genes encoding components of the pre-BCR. This receptor is composed of a functionally rearranged IgH, the surrogate light chains Λ5 and VpreB, and appears to be important for the progression of the pre-B cell stage and initiation of Ig light chain (IgL) gene recombination (23). The pre-BCR also contains the Ig-associated α and β proteins that together with the B lymphoid kinase (Blk) transduce signals through the receptor (24). Blk is a Src family kinase expressed throughout B cell development with the exception of the plasma cell stage (25–27). The Blk gene is under the control of a weak promoter (26, 28) containing a functionally important binding site for the paired domain transcription factor B cell-specific activator protein (BSAP; Pax-5) (28, 29). Furthermore, ectopic expression of BSAP activates the promoter in B and plasma cell lines (30), supporting the idea that BSAP participate in the regulation of the Blk promoter. The BSAP binding site overlaps with a binding site for NF-kB transcription factor complexes, and induction of NF-kB has also been shown to correlate with induction of the endogenous Blk gene (30). The expression of Blk and BSAP are largely overlapping (28), further supporting that BSAP is a regulator of Blk expression. However, mice carrying a homologous disruption of the BSAP-encoding Pax-5 gene still develop pre-B cells expressing Blk transcripts (31, 32). This implies that other yet unidentified transcription factors have the ability to compensate for BSAP in the function of the Blk promoter in early B cell development. One such candidate could be EBF, expressed in pre-B cells from BSAP-deficient mice (31, 32).

We here present data proposing that EBF interacts with the Blk promoter in vivo as well as in vitro and has the ability to activate the promoter in nonlymphoid HeLa cells. We also show data indicating that the EBF binding site is important for full function of the Blk promoter in pre-B cell lines. Furthermore, coexpression of EBF and BSAP in HeLa cells resulted in a more than additive effect on the activation of the Blk promoter, suggesting that BSAP and EBF cooperate. These results imply that EBF is involved in the regulation of the Blk gene, which offers a possible explanation to

1 Abbreviations used in this paper: EBF, early B cell factor; Blk, B lymphoid kinase; BSAP, B cell-specific activator protein; IgH, Ig heavy chain; BCR, B cell receptor; IgL, Ig light chain; β-gal, β-galactosidase; TBE, Tris-borate, EDTA; Blk-EBF, Blk promoter with mutated EBF binding site; Blk-BSAPM binding site, Blk promoter with mutated BSAP binding site; Blk-EBM, Blk promoter with double mutated EBF

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the finding that the Blk gene is active in BSAP-deficient mice and defines a genetic target for EBF.

Materials and Methods

Tissue culture conditions

All cells were grown in RPMI 1640 medium supplemented with 7.5% FCS, 10 mM HEPES, 2 mM pyruvate, 50 μM 2-ME, and 50 μg gentamicin per ml (complete RPMI 1640 media) (all purchased from Life Technologies, Täby, Sweden) at 37°C and 5% CO₂.

In vivo footprinting analysis

In vivo footprinting analysis was based on a modified linker-mediated PCR (33, 34). Briefly, 50 million cells were incubated in 1 ml RPMI 1640 medium supplemented with 0.1% dimethyl sulfate for 5 min, after which 25 ml of ice-cold medium was added. The cells were then washed, and DNA was extracted and boiled in pipediner. The denatured DNA was annealed to Blk anti-sense oligo 1, and dsDNA was synthesized by extension with Klenow enzyme. The sample was then ligated to a double stranded linker of oligo T1 and B1. The ligated DNA was then amplified by PCR using oligo T1 and Blk anti-sense 2, and the PCR product was labeled by linear PCR with [γ-32P]ATP-labeled oligo Blk anti-sense 3. The labeled sample was then separated on a 6% sequencing gel. A detailed protocol is available upon request.

Oligonucleotides used for in vivo footprinting analysis included the following: Blk anti-sense 1 (5'-CCGGCATGAAACGGCTCTAAGAC), T1 (5'-GGCGGATCGGCAGTATGATC), Blk anti-sense 2 (5'-AGGGTTTTTCATGGAATGGTATCAGT), and Blk anti-sense 3 (5'-GTTCTAGGCGACCTGACCTGAC).

Transient transfections and luciferase assays

A total of 250,000 HeLa cells were grown overnight in 1 ml of complete RPMI 1640 medium in a 24-well plate. The cells were washed once with serum-free medium (OPTIMEM; Life Technologies), and 800 μl of serum-free medium (OPTIMEM; Life Technologies) was added for transfection. Then, 5 ml (Promega), which is hydrolysed to yellow o-nitrophenol by o-galactosidase (β-gal), was then measured in a spectrophotometer at 405 nm. Luciferase assays were performed with a luciferase assay kit (Promega) using 20% of six-well plates for 48 h. Preparation of protein extracts and luciferase analysis of transfected cells were cultured in 5 ml of complete RPMI 1640 medium in a 24-well plate. The cells were washed once with 25 ml of ice-cold medium was added. The cells were then harvested after 40 h, and protein extracts were prepared directly in the 24-well plates using 80 μl of cell lysis buffer (Promega, Falkenberg, Sweden). This procedure result in protein extracts of high quality and the need to normalize for the protein content in the extracts by cotransfection of a β-galactosidase reporter (M.S., unpublished observation). Luciferase assays were then conducted with 20 μl of the obtained extracts and 200 μl of luciferase assay reagent (Promega).

All other cell lines were washed twice in TBS (140 mM NaCl, 5 mM KCl, 25 mM Tris, pH 7.4, 0.6 mM phosphate, 0.5 mM MgCl₂, 0.7 mM CaCl₂), and 2.5 × 10⁶ cells were transfected with 2 μg reporter gene constructs in 0.65 ml TBS with 0.7 mg DEAE-dextran (Pharmacia, Uppsala, Sweden) per ml for 30 min at 20°C. After a single wash in TBS, the transfected cells were cultured in 5 ml of complete RPMI 1640 medium in six-well plates for 48 h. Preparation of protein extracts and luciferase assays were performed with a luciferase assay kit (Promega) using 20% of the total protein extract. The obtained luciferase activity was normalized against the activity of a cotransfected CMV-controlled β-galactosidase (β-gal) reporter gene. β-Gal activity was measured by incubation of 20% of the protein extracts with colorless (o-nitrophenyl-β-D-galactopyranosid) (Promega), which is hydrolysed to yellow o-nitrophenol by β-gal. The OD was then measured in a spectrophotometer at 405 nm.

In vitro transcription and translation

Recombinant proteins were generated by coupled in vitro transcription-translation by using a reticulocyte lysis kit (Promega). Then, 5 μl of a 25-μl reaction mix was used for EMSAs.

Results

EBF interacts with the Blk promoter

Inspection of the minimal Blk promoter region (26, 28) identified a potential binding site for EBF 15 bp upstream the most 5'-flanking sequence (Fig. 1A). This site had one mismatch as predicted by a computer program (30), together with the Blk antisense primer. The Blk promoter cloned in pGL3 was then amplified by PCR using the Blk sense and anti-sense primers, followed by digestion with the restriction enzyme HindIII and fill-in labeled with [γ-32P]ATP by incubation with T4 polynucleotide kinase (Boehringer Mannheim, Mannheim, Germany), annealed, and purified on a 6% polyacrylamide TBE gel, which was dried and subjected to autoradiography. Competitors were added at molar excesses indicated in the respective figures.

Methylation of 5'-[32P]-labeled oligonucleotides was performed as described (36). Methylation DNA was incubated with in vitro-transcribed/translated EBF or nuclear extracts in a 3-fold up-scaled EMSA, and the obtained complexes were separated by electrophoresis through a 6% polyacrylamide gel. The bands representing bound and free DNA were then excised, extracted in TE (10 mM Tris-HCl, pH 7.6, 1 mM EDTA), ethanol precipitated, boiled in 1 M pipediner for 25 min, lyophilized three times, resuspended in sequence loading buffer, and separated on a 10% sequencing gel.

Oligonucleotides used for EMSAs were as follows: Blk sense (5'-ACCCAAAAGGCATGAAAGAAATTGGT), Blk anti-sense (5'-CAAGTGTTTCTCATTGATTTTGTTGTT), Blk-EBFM sense (5'-GAGGAGACCTCAAGGGATTTGGG), and Blk anti-sense (5'-GGAGACCTGACGGTAGCTTCAGCTTGC).

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black dots in Fig. 1, A and B, within the potential EBF binding site. This suggested that these residues were protected from methylation in vivo, indicating that the potential EBF binding site was occupied. In contrast, no clear methylation protection could be detected within the BSAP binding site. To investigate if EBF could interact with the site in vitro, we synthesized an oligonucleotide spanning the region between −32 and −7 of the Blk promoter and used this as a binding site for recombinant in vitro-translated mouse EBF in an EMSA (Fig. 1C). Even though two bands could be detected when large amounts of unprogrammed reticulocyte
lysate was included, the use of EBF-programmed lysate resulted in one prominent complex that was not present in unprogrammed reticulocyte lysate. This suggests that recombinant EBF has the ability to interact with the Blk promoter. To further examine the formed complex, we competed for the formation of the Blk promoter/EBF complex by addition of duplex oligonucleotides spanning either the Blk (Bik-EBF) or mb-1 (mb-1-EBF) promoter EBF sites or a consensus octamer site (OCT). Both the Blk and the mb-1 EBF binding sites competed for EBF binding but the affinity of the former appeared to be about 5-fold lower than that of the mb-1 promoter. The octamer-containing oligonucleotide did not compete for binding proposing that the interactions were specific. These data indicate that the Blk promoter contains an EBF binding site occupied in vivo in a pre-B cell line.

**EBF induces Blk promoter activity in a nonlymphoid cell line**

To define the EBF binding site in the context of the intact promoter and to generate an EBF mutant Blk promoter (Fig. 2A), we used PCR to generate wild-type (−85 to +152) or EBF-mutated Blk promoters. This 237-bp region has been shown to contain transcription initiation sites and the majority of the functional activity of the Blk promoter (26, 28). The wild-type (Blk) and EBF-mutated Blk (Blk-EBFM) promoters or the λ5 promoter (15) were then used as competitors for binding of recombinant EBF to the Blk EBF site in an EMSA (Fig. 2B). Both the wild-type Blk and the λ5 promoters competed efficiently for binding of recombinant EBF, while the Blk promoter containing point mutations in the EBF site competed for EBF binding with a reduced affinity (Fig. 2B). The fact that an EBF site-mutated Blk promoter still competed weakly for EBF binding indicates that other, low-affinity EBF binding sites may be present in the promoter. One potential site could be found in position +61 to +74 (Fig. 1A), but the location and the low affinity discouraged us to investigate this second potential EBF binding site any further. To examine whether the Blk promoter EBF binding site was able to confer EBF-induced functional activity, we made transient transfections into nonlymphoid HeLa cells, with luciferase reporter constructs controlled by either the wild-type or the EBF-mutated Blk promoter. (Fig. 2C). The reporter plasmids were cotransfected with either empty expression vector (cDNA3) or with the same vector containing an EBF-encoding cDNA. Inclusion of EBF expression plasmid resulted in a 9-fold induction of the wild-type Blk promoter (Blk), while the same amount of EBF expression plasmid only resulted in a 2.5-fold induction of the EBF site-mutated Blk promoter (Blk-EBFM).

A fos basal promoter was induced 1.5-fold, and a λ5 promoter was induced 16-fold in the same experiment (data not shown). This indicates that the interaction of EBF with the defined binding site in the Blk promoter induces functional activity of the promoter in nonlymphoid cells.

**The Blk promoter EBF site interacts with EBF-like proteins in nuclear extracts from pre-B cell lines**

To examine if EBF interacted with the Blk promoter in pre-B cells, we used EMSA to study the factors bound to a Blk promoter EBF site (−32 to −7) in a 230–238 pre-B cell nuclear extract (Fig. 3A). This resulted in two bands of which that with the lowest mobility (C1) was competed for binding by the Blk EBF site (Blk-EBF) as well as by the mb-1 EBF site EBF site (mb-1 EBF), but not by the octamer (OCT) containing control oligonucleotide. The high-mobility complex (C2) was only competed efficiently for binding by the Blk EBF site, suggesting that this complex is unrelated to EBF. The band with the lowest mobility was also efficiently competed for binding by the full-length Blk (Blk) and the λ5 promoters but not by the Blk promoter carrying a mutation in the EBF site (Blk-EBFM). The faster migrating complex was competed for binding by both the wild-type and the EBF-mutated Blk promoter but not by the λ5 promoter, suggesting that this complex was formed by protein interaction with a region distinct from the EBF binding site. To confirm this, and to more carefully examine if the low-mobility complex indeed was identical with EBF, we made methylation interference experiments with the Blk promoter EBF binding site and recombinant in vitro-translated EBF (rEBF) or nuclear extracts from 230–238 pre-B cells (Fig. 3B). Unbound DNA generated the expected G ladder while recombinant EBF and C1 as well as C2 appeared to be sensitive to methylation in the two central G residues in the sense strand. A different pattern of interaction was obtained using the anti-sense strand where both binding of recombinant EBF and C1 was sensitive to methylation of three of the G residues within the defined EBF site, while this was not the case for the unidentified low-mobility complex C2. To further characterize the two complexes formed by interaction of nuclear factors with the Blk promoter, we performed EMSA using the
oligonucleotide covering the region \( -7 \) to \( -32 \) as above (Fig. 4) and nuclear extracts from a panel of cell lines. An octamer containing oligonucleotide (OCT), a full-length \( \text{Blk} \) promoter (Blk), a full-length EBF-mutated \( \text{Blk} \) promoter (Blk-EBFM), and a full-length \( A5 \) (A5) promoter as indicated. F indicates the free probe. B. Methylation interference analysis of the binding of recombinant in vitro-translated EBF (rEBF) and nuclear factors (C1, C2; A) interacting with the \( \text{Blk} \) promoter EBF site. The complexes obtained with a pre-methylated \( \text{Blk} \) EBF site oligonucleotide were cut out of the gel, and the DNA was eluted, digested by piperidine boiling, and separated on a sequencing gel. The G ladders obtained with unbound DNA (Free) is compared with those obtained from the different complexes (rEBF, C1, C2). The positions of the G residues are shown by the DNA sequence, and residues essential for the binding of EBF are indicated by a dot.

**FIGURE 3.** The EBF binding site in the \( \text{Blk} \) promoter interacts with EBF in a pre-B cell nuclear extract. A, EMSA with a \( \text{Blk} \) EBF site probe and nuclear extracts from 230–238 pre-B cells. The obtained complexes were competed for binding by the \( \text{Blk} \) EBF binding site (Blk-EBF), the \( mb-1 \) promoter EBF bindings site (mb-1-EBF), an octamer containing oligonucleotide (OCT), a full-length \( \text{Blk} \) promoter (Blk), a full-length EBF-mutated \( \text{Blk} \) promoter (Blk-EBFM), and a full-length \( A5 \) (A5) promoter as indicated. F indicates the free probe. B. Methylation interference analysis of the binding of recombinant in vitro-translated EBF (rEBF) and nuclear factors (C1, C2; A) interacting with the \( \text{Blk} \) promoter EBF site. The complexes obtained with a pre-methylated \( \text{Blk} \) EBF site oligonucleotide were cut out of the gel, and the DNA was eluted, digested by piperidine boiling, and separated on a sequencing gel. The G ladders obtained with unbound DNA (Free) is compared with those obtained from the different complexes (rEBF, C1, C2). The positions of the G residues are shown by the DNA sequence, and residues essential for the binding of EBF are indicated by a dot.

The EBF binding site is important for the full activity of the \( \text{Blk} \) promoter in pre-B cell lines

To investigate the importance of an intact EBF binding site for the function of the \( \text{Blk} \) promoter in cells of the B lineage, we introduced mutations in the EBF and/or BSAP binding sites. The introduced BSAP mutation was identical with the mutation B suggested to have the most dramatic effect on \( \text{Blk} \) promoter function (30) in B cells and to prevent binding of BSAP to the defined BSAP binding site (28, 30). To examine the effects of the introduced mutations on protein binding, we used EMSA with a probe spanning a region between \( -285 \) to \( -70 \) of the \( \text{Blk} \) promoter that includes both the BSAP and the EBF binding site and nuclear extracts from 230–238 pre-B cells. This resulted in three major complexes of which the one with lowest mobility was competed for binding by both the \( \text{Blk} \) (Blk-EBF) and the \( mb-1 \) EBF (mb-1-EBF) binding sites but not by the BSAP binding site from the human \( CD19 \) promoter (CD19-BSAP) (39) (Fig. 5A). The complex with intermediate mobility was only competed for binding by the \( CD19 \) BSAP binding site while the fastest migrating complex was competed for binding only by the \( \text{Blk} \) EBF binding site. This
suggests that the low-mobility complex was formed due to interaction of the Blk promoter with EBF, the medium-mobility complex was formed due to interaction with BSAP, and the fastest-migrating complex was formed due to interaction with factor C2 (Fig. 3). All three observed complexes were competed for binding by a full-length wild-type Blk promoter (Blk), while the low-mobility complex was not competed for binding by a EBF-mutated Blk promoter (Blk-EBFM) (Fig. 5B). The Blk promoter containing the BSAP mutation (Blk-BSAPM) showed only marginally reduced BSAP binding, suggesting the existence of other BSAP binding sites in the Blk promoter. The promoter carrying mutations in both the EBF and BSAP binding site (Blk-EBM) competed with a slightly reduced efficiency for BSAP and a dramatically reduced efficiency for EBF. Both the low- and intermediate- but not the high-mobility complexes competed for binding by the l5 promoter, known to interact with both EBF (15) and BSAP (40). This suggests that in pre-B cells, both EBF and BSAP interact independently with the Blk promoter. To investigate the function of the EBF binding site in the Blk promoter, we made transient transfections of reporter constructs controlled by either a wild-type or the mutated Blk promoters into cells of the B lineage. The activities of these constructs were low (26, 30) (data not shown), which led us to introduce an IgH intron enhancer (E\textscript{M} 3\textprime) of the luciferase gene. This resulted in an increase of activity, which allowed us to analyze the promoter function in the pre-B cell lines 18–81 and 230–238 and in the B cell line A20 (Fig. 5C). The introduction of mutations in both sites (Blk-EBFM) resulted in a reduction of the functional activity to 25–30% of that of the wild-type Blk promoter (Blk) in pre-B cell lines, while no significant effect could be observed in the A20 B cells. Furthermore, the EBF binding site mutation did not effect the basal function of the promoter in the non-EBF expressing S194 plasmacytoma cell line or in HeLa cells (data not shown). The opposite was observed after mutation in the BSAP site (Blk-BSAPM), which had only a marginal effect on the function of the Blk promoter in the pre-B cells but resulted in a 50% reduction of activity in the A20 B cells. Introduction of mutations in both sites (Blk-EBM) did not reduce the functional activity of the promoter below the level observed with an EBF mutation in the pre-B cells, while a marginal effect could be observed in the A20 cells. This suggests that a functional EBF binding site is important for the full activity of the Blk promoter in pre-B cells but not in more mature A20 B cells.

**EBF and BSAP activate the Blk promoter cooperatively**

The ability of the Blk promoter to interact with both EBF and BSAP is shared by a large number of genes expressed early in the B cell lineage (13, 15, 16, 18, 40–42). This opens the possibility that cooperative activation by EBF and BSAP may be a common theme in B cell-specific gene regulation. Because the mutation of the defined BSAP binding site did not significantly effect the binding of BSAP to the intact Blk promoter, we decided to do reconstitution experiments to investigate a potential cooperation between EBF and BSAP. Thus we made transient transfections of Blk promoter controlled reporter constructs, together with expression plasmids encoding either EBF or BSAP or the combination of the two plasmids into HeLa cells (Fig. 6). Transfection of the Blk promoter-controlled reporter construct with increasing amounts (50–200 ng) of BSAP expression plasmid resulted in up to a 10-fold increase of reporter activity, while 400 ng of EBF expression...
plasmid resulted in a 9-fold increase of promoter activity. Transfection of 50, 100, or 200 ng BSAP expression vector together with 400 ng EBF expression plasmid resulted in a 12-, 18-, or 35-fold induction of reporter activity. This effect could not be explained by alterations in levels of the transcription factors because EMSA analysis from single- and double-transfected cells suggested that they expressed equal amounts of EBF and BSAP (data not shown). The importance of a functional EBF binding site for the cooperative action of EBF and BSAP was investigated by transfection of a reporter gene under control of an EBF binding site-mutated Blk promoter (Blk-EBFM; Fig. 2) and expression plasmids encoding EBF or BSAP. Inclusion of 200 ng BSAP expression plasmid induced the reporter gene 7.5-fold (Fig. 6), while the combination of 200 ng BSAP and 400 ng EBF expression plasmids increased the reporter activity 12-fold (Fig. 6). Transfection of a minimal TATA box controlled reporter gene together with 400 ng EBF and 200 ng of BSAP expression plasmid resulted in a 2.4-fold up-regulation of promoter activity (data not shown). These data suggest that both EBF and BSAP interact with, and has the ability to activate, the Blk promoter independently, but that the presence of both factors results in a functional cooperation dependent of the EBF binding site.

Discussion

Our analysis of the Blk promoter suggests that EBF interacts with a region occupied in vivo and has the ability to activate the promoter in nonlymphoid HeLa cells. Furthermore, we present...
studies of the initiate a full B cell developmental program. Rather, it appears that absence of EBF. Hence, it is uncertain if EBF alone is able to deficient mice do develop a pro-B cell compartment, suggesting solely dependent on the expression of EBF. Furthermore, EBF- indicating that the activation of genes in the 70Z/3 cells is not ectopic expression of EBF in the hemopoietic cell line Ba/F3 (15), pre-B cell stage and thus suggest that EBF could be a fate-deter- ability to activate a large majority of the genes known to define the cells have been unsuccessful. We believe that this is due to the topic expression of EBF in the bone marrow-derived cell clone Blk promoter. This functional cooperation between EBF and BSAP in activation of the Blk promoter.

EBF has previously been shown to interact with functionally important regions of the mb-1 (6, 13), B29 (14), L5 (15, 16), and VpreB (15, 18) promoters, and now also of the Blk promoter. This suggests that EBF is a pleiotropic activator of genes encoding components of the pre-BCR complex. EBF has also been reported to activate the expression of IgL genes and BSAP in dedifferen- tiated 70Z/3 pre-B cells (17). This would propose that EBF has the ability to activate a large majority of the genes known to define the pre-B cell stage and thus suggest that EBF could be a fate-determin- ing factor in B cell development. However, the activation of some of the suggested target genes was not observed upon stable ectopic expression of EBF in the hemopoietic cell line Ba/F3 (15), indicating that the activation of genes in the 70Z/3 cells is not solely dependent on the expression of EBF. Furthermore, EBF- deficient mice do develop a pre-B cell compartment, suggesting that the earliest stages of B cell development occur even in the absence of EBF. Hence, it is uncertain if EBF alone is able to initiate a full B cell developmental program. Rather, it appears that the factor is involved in the regulation of a large number of genes important to define the B cell lineage and that the role of EBF is to make B cell differentiation possible rather than to determine the fate of the progenitor cells.

The idea that EBF and BSAP act in concert to promote B cell differentiation and share target genes has now been supported from studies of the mb-1 (13, 42), L5, VpreB (40, 41), and Blk (28, 30) (this report) promoters. However, such a cooperation has not been clearly shown experimentally. We here present data suggesting that these factors indeed interact functionally to activate the Blk promoter. This functional cooperation did involve some synergy because the combined effect was about twice as high as the expected additive effect. The combined activity was also to a large extent dependent on a functional EBF binding site, because the induction of the mutated Blk promoter after coexpression of the proteins was largely impaired. Mutation of the defined BSAP site did not significantly effect EBF binding to the whole promoter. However, this mutation did prevent binding to a factor, presumably NF-κB (30), in A20 cells (P.A., unpublished observation). This factor could not be detected in pre-B cells, suggesting that the effect of this mutation on promoter function in A20 cells (30) may be a result of impaired binding of this factor rather than BSAP. However, previous studies indicate that several mutations in the BSAP site of the Blk promoter results in functional reductions of promoter activity (30); thus, we believe that both EBF and BSAP participate in the regulation of the Blk promoter. Similar findings have been obtained from experiments addressing the regulation of the mb-1 promoter, where mutation of either the EBF (13) or the BSAP (42) binding sites results in reduced functional activity of the promoter. However, the observations that mutation of either of the binding sites only results in reductions, as opposed to a com- plete abolishment, of promoter activity, may suggest a partial re- dundancy among the two proteins. A degree of redundancy is also supported by the finding that either of the factors can activate the Blk promoter in HeLa cells.

It is also interesting to note that the functional relevance of the Blk EBF binding site appears to be restricted to the pre-B cell stage. Similar observations has been made in studies of EBF reg- ulation of the B29 (14, 43) and mb-1 (13, 44) genes, questioning the role of EBF in mature B cells. This may be relevant because EBF has been suggested to directly regulate the expression of the pre-B cell-specific genes encoding the surrogate light chains L5 and Vpre-B (15, 16, 18). The finding that the expression pattern of these genes (22, 45) do not totally correlate to that of EBF, because EBF can be expressed also in B cells (6, 13), has lead to the suggestion that EBF alone cannot mediate pre-B cell-specific ex- pression of the surrogate light chain genes. However, this could be achieved if EBF function is altered in the B cell, either by post- translational mechanisms or due to the lower expression levels observed in B as compared with pre-B cells (6, 13). A third ex- planation could be expression of a protein that interacts with EBF to modify its function or target gene specificity. Such a mechanism has been proposed from studies of the transcriptional regulation of olfactory neuron-specific genes where the Zn finger protein Roaz modulates the activity of EBF (Olf-1) (46, 47). The continued expression of EBF target genes in more mature developmental stages may then be achieved by a developmental shift to involve other transcription factors (43).

Our attempts to activate the endogenous Blk gene by stable ectopic expression of EBF in the bone marrow-derived cell clone Ba/F3 or by transient expression of EBF in S194 plasma-cytoma cells have been unsuccessful. We believe that this is due to the combined effects of a single EBF binding site and the rather low functional activity of the Blk promoter. The modest activity of the Blk promoter (28) (this study), contrasted by rather high expression levels of the endogenous gene (26, 28), may suggest this gene to be under the control of a distal enhancer element regulated by other factors than BSAP or EBF. This element could then together with EBF compensate for the absence of BSAP in mice carrying a homologous disruption of the Pax-5 gene (31).

Flow cytometric analysis of bone marrow from EBF−/− mice suggested the presence of cells expressing B220 and CD43 but not BP1 or HSA (9). PCR analysis showed expression of sterile Ig transcripts (Iγ, and μ0), TdT, and IL-7 receptor transcripts but
neither mRNA for Rag-1, Igα, VpreB, A5, Igβ, nor Ig gene recombination was detected (9). This suggests that in the absence of EBF, B cell development is arrested at the transition from fraction A (pro-B) to fraction B pre-B cells. This phenotype cannot be explained by the absence of any of the formerly known genetic targets and neither do we believe that the absence of Blk can explain the observed phenotype. Thus, we suggest that EBF is involved also in the regulation of other yet unidentified genes whose identity and function will increase the understanding of early B cell development.

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References