

Erythroid Krüppel-Like Factor Directly Activates the Basic Krüppel-Like Factor Gene in Erythroid Cells[∇]

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The Sp/Krüppel-like factor (Sp/Klf) family is comprised of around 25 zinc finger transcription factors that recognize CACCC boxes and GC-rich elements. We have investigated basic Krüppel-like factor (Bklf/Klf3) and show that in erythroid tissues its expression is highly dependent on another family member, erythroid Krüppel-like factor (Eklf/Klf1). We observe that *Bklf* mRNA is significantly reduced in erythroid tissues from *Eklf*-null murine embryos. We find that *Bklf* is driven primarily by two promoters, a ubiquitously active GC-rich upstream promoter, 1a, and an erythroid downstream promoter, 1b. Transcripts from the two promoters encode identical proteins. Interestingly, both the ubiquitous and the erythroid promoter are dependent on Eklf in erythroid cells. Eklf also activates both promoters in transient assays. Experiments utilizing an inducible form of Eklf demonstrate activation of the endogenous *Bklf* gene in the presence of an inhibitor of protein synthesis. The kinetics of activation are also consistent with *Bklf* being a direct Eklf target. Chromatin immunoprecipitation assays confirm that Eklf associates with both *Bklf* promoters. Eklf is typically an activator of transcription, whereas Bklf is noted as a repressor. Our results support the hypothesis that feedback cross-regulation occurs within the Sp/Klf family in vivo.

The mammalian Sp/Krüppel-like factor (Sp/Klf) family consists of 9 Sp and 17 Klf subfamily proteins (78, 88). Sp/Klfs bind GC-rich elements and related CACCC sequences in DNA by means of three tandem C₂H₂ zinc fingers found at or near their C termini (27, 46, 75). While this zinc finger domain is highly conserved among family members (85), other regions of the proteins are not. Accordingly, although they recognize similar DNA-binding sites, some Sp/Klfs, such as erythroid Klf (Eklf)/Klf1, serve predominantly as activators (56); others, such as basic Klf (Bklf)/Klf3, are regarded as transcriptional repressors (84). It is possible, however, that many and perhaps all Sp/Klfs function as either activators or repressors, depending on promoter and cellular context (38). Sp/Klf proteins have been found to play critical roles in a diversity of biological processes (9, 21), including erythropoiesis (8, 53, 63, 68, 87, 91), adipogenesis (7, 58, 64), and carcinogenesis (10, 29, 61, 90).

Many Sp/Klfs, such as Sp1, are broadly expressed, whereas others, such as Eklf, exhibit more-limited expression patterns. Eklf is so named because its expression is restricted mainly to erythroid cells, but it is also detectable in macrophages and mast cells (52, 56). It is a potent transcriptional activator recognized for binding CACCC boxes and related sequences that fall into the general consensus sequence 5'-NCNCNCCCN-3' (27). These motifs are found in the regulatory regions of many

erythroid genes (27, 56, 71). The adult β -globin promoter, for instance, contains an Eklf recognition site that is critical for β -globin expression (23, 27). As a result, *Eklf* knockout mice die at around embryonic day 15 (E15) with a severe β -globin deficiency (63, 68).

Efforts have been made to rescue the *Eklf*-null phenotype by the overexpression of human γ -globin. However, despite a correction of the globin chain imbalance, the null mice still die in utero due to hemolysis (67). This result suggests that other critical Eklf target genes exist. Recently, several genes have been shown to be underexpressed in *Eklf*-null fetal liver (24, 33, 70). These include the genes for α -hemoglobin stabilizing protein (Ahsp), which binds and stabilizes free α -hemoglobin and prevents its cytotoxic precipitation, and dematin, a cytoskeletal protein required for membrane integrity in erythrocytes. Numerous other Eklf target genes have been proposed from studies of differential expression in *Eklf*-null fetal liver. However, in many cases, it is uncertain whether these are direct targets or whether they are genes that are indirectly influenced by hypoxia, incomplete erythroid maturation, or skewed cellular heterogeneity in the *Eklf*-null mice.

Work with physiologically relevant cell lines has been employed to circumvent these complexities. The B1.6 cell line (17), for instance, has allowed for the validation of *Ahsp* and *dematin* as direct targets of Eklf (33). This line was generated by immortalizing E14.5 *Eklf*-null fetal liver erythroid progenitors using the J2 retrovirus. The cells were then rescued using a transgene encoding Eklf fused to the ligand-binding domain of the estrogen receptor Eklf-ER (17, 50). A mutant form of the ER was used, so that the fusion would respond to the synthetic steroid tamoxifen but not to estrogen, which may be present in culture medium (50). The addition of tamoxifen results in the accumulation of Eklf-ER in the nucleus, the

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activation of Eklf target genes, and the subsequent hemoglobinization and terminal differentiation of the cells (17).

It has long been noted that there are several apparently abundant CACCC box-binding proteins present in erythroid cells, in addition to Eklf and the ubiquitous protein Sp1 (19, 32). A cDNA-encoding Bklf was isolated using probes specific for the zinc finger domain of Eklf and Sp1 and relaxed-stringency hybridization with a mouse erythroleukemia (MEL) cDNA library (20). Bklf is found in a wide range of tissues and cell types but is particularly abundant in hematopoietic tissue (20). Like Eklf, Bklf binds CACCC motifs in preference to GC boxes (20). Bklf has been shown to bind *in vitro* the promoter CACCC elements of many erythroid genes, such as adult β -globin, fetal $A\gamma$ -globin, *Gata-1*, *carbonic anhydrase I*, *porphobilinogen deaminase (Pbgd)*, and *pyruvate kinase* (20, 66). The CACCC-binding activity of Bklf is more readily detected in yolk sac and fetal liver than that of Eklf (20), suggestive of a potential role in hematopoiesis. The phenotype of *Bklf*-null mice, however, is complex, consistent with Bklf being broadly expressed. Precise target genes and biological roles for the protein have been difficult to define (unpublished results).

Functionally, Bklf was initially shown to be a transcriptional activator. However, transactivation was observed only with high levels of Bklf and was not as strong as Eklf-mediated activation (20). Bklf has since been established as a potent transcriptional repressor in many systems (66, 84, 86). The protein is highly basic, hence the name basic Krüppel-like factor, and it includes a repression domain that contains a Pro-Val-Asp-Leu-Thr motif through which Bklf interacts with the corepressor C-terminal binding protein (84).

It has previously been noted that Bklf protein levels are reduced in the fetal liver (erythroid tissue) but are unaffected in the fetal brain (nonerythroid) in *Eklf*^{-/-} mice (20, 68). Whether this is a direct or indirect effect has not previously been explored. We have now found that *Bklf* expression is driven from two promoters. The upstream promoter, 1a, is active in a wide range of tissues, while the downstream promoter, 1b, which gives rise to a novel *Bklf* transcript (but encodes an identical protein), is active predominantly in erythroid tissues. We find that transcripts from both promoters are underexpressed in *Eklf*-null fetal liver. Activation of both promoters by Eklf is observed for heterologous cells in transient transfection experiments. Chromatin immunoprecipitation experiments confirm that Eklf-ER directly binds to both promoters in B1.6 cells. The induction of Eklf-ER activates the endogenous downstream erythroid promoter 1b in the B1.6 line but, unexpectedly, no activation of the upstream promoter is observed with this system. The kinetics of the induction of promoter 1b and the observation that induction occurs in the presence of an inhibitor of protein synthesis further suggest that *Bklf* is a direct Eklf target gene.

We have also examined several other *Klf* genes and, interestingly, find that *Tieg/Klf10* is also activated by Eklf-ER in B1.6 cells in the presence of a protein synthesis inhibitor and is underexpressed in *Eklf*-null fetal liver. These results reveal cross-regulation of the Klf subfamily in erythroid cells.

MATERIALS AND METHODS

Genotyping. Genotyping of the *Eklf*-null mice was performed as described previously (33).

RNA extraction. E14.5 fetal livers and tissue sections (up to 140 mg) from 13- to 16-week-old female C57BL/6 mice (68) were freshly homogenized for RNA extraction after washing with phosphate-buffered saline (Sigma, St. Louis, MO). Adult mice were sacrificed by cervical dislocation to avoid indirect effects caused by terminal anesthesia. Total RNA was extracted with TRI REAGENT (Sigma) per the supplier's protocol but with an additional centrifugation step at 12,000 × g for 10 min at 4°C following homogenization to reduce contamination by genomic DNA. RNA was then cleaned up with RNeasy kits (QIAGEN Pty. Ltd., Victoria, Australia) and was subsequently subjected to DNase treatment with a DNA-free kit (Ambion, Austin, TX) as instructed by the supplier.

Real-time RT-PCR. Up to 5 μ g of RNA was used as a template for cDNA synthesis primed by random hexamers with a SuperScript III First Strand cDNA Synthesis kit (Invitrogen, Carlsbad, CA). Quantitative real-time reverse transcription (RT)-PCR was performed with SYBR Green PCR Master Mix by using an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA). Approximately 10 ng of cDNA, as estimated by UV spectrophotometry, was used in each RT-PCR. Samples were normalized with respect to 18S rRNA levels. Reactions containing serial dilutions of gel-purified amplicon were included in each RT-PCR run in order to construct standard curves for relative quantification. Minus RT and no-template reactions were included as negative controls. The analysis of real-time PCR data was done with ABI Prism 7000 sequence detection system software (version 1.1).

Real-time PCR primers. Paired primers were designed with PrimerExpress software (Version 2.0) (Applied Biosystems) to cross, where possible, exon-exon boundaries to prevent amplification of any contaminating genomic DNA. Primer sequence specificity was checked using the Basic Local Alignment Search Tool (BLAST; <http://www.ncbi.nlm.nih.gov/BLAST>) (4). For each primer set, optimal reaction concentrations, ranging between 200 nM and 600 nM, were determined. The sequences of forward and reverse primers used are as follows: 18S, 5'-CA CGGCCGGTACAGTGAAAC-3' (forward) and 5'-AGAGGAGCGAGCGAC CAA-3' (reverse); *Bklf* exons 4/5, 5'-GAAATGTCACCCCTTAATGAAC-3' and 5'-CACGATGACGGAAGGATGGT-3'; *Bklf* exons 1a/2, 5'-CGGGCCTG GGTTCCTTG-3' and 5'-GATCAAACATGAGCATCCTTTCAG-3'; *Bklf* exons 1b/2, 5'-GGTGAATTCTGTTTCAGTCAAC-3' and 5'-CCACGCTTC TAGGGTGTCT-3'; *Klf10*, 5'-GCAGCCAACCATGCTCAAC-3' and 5'-CCC CTCTGTGGCTTTTCAG-3'; *Klf13*, 5'-CGAGAAAGTTTACGGGAAATCT TC-3' and 5'-CAGCGAAAGGCTCTCA-3'; and *Klf16*, 5'-TCACACCTGC GGACTACA-3' and 5'-CAGAACGGGCGAACTTCTTG-3'.

EMSA. Nuclear extracts were prepared and electrophoretic mobility shift assays (EMSAs) were carried out as described previously (20). Anti-Eklf and anti-Bklf antibodies have been described previously (20). Anti-Sp1 and anti-Sp3 antibodies were supplied by Santa Cruz Biotechnology, Santa Cruz, CA.

Oligonucleotides used in the synthesis of radiolabeled probes are as follows: mouse β -major globin CACCC probe, 5'-TAGAGCCACACCTGGTAAG-3' and 5'-CTTACCAGGGTGTGGCTCTA-3'; *Bklf* promoter 1b CACCC probe, 5'-AGTACTGGGTGTGGCAGAATCTTATCTGAAGCT-3' and 5'-AGCT TCAGATAAGATTCTGCCACACCCAGTACT-3'.

5' RACE. One μ g MEL total RNA was used as a template for first-strand cDNA synthesis with a SMART rapid amplification of cDNA ends (RACE) cDNA amplification kit (BD Biosciences, Palo Alto, CA) as instructed by the supplier. RACE PCR was performed as described in the Advantage 2 Polymerase kit manual (BD Biosciences) with an additional five repetitions of the final cycle. For each reaction, cDNA synthesized from ~20 ng total RNA was used. 5' RACE was primed with an oligonucleotide, 5'-TCCACCGCTCCACCTGTA TCCC-3', specific for the third exon of *Bklf*. Confirmatory 5' RACE PCR was conducted with a primer, 5'-GGGACTGGATCAAACATGAGCATCCTT-3', specific for exon 2 of *Bklf*. 3' RACE primers, specific for *Bklf* exon 1b, were as follows: 5'-ATTGCATCCCATCTGAAGCCAAGC-3' and 5'-GAGAGGCAC AGATTCCGAAATATCCG-3'. RACE products were resolved by 2% agarose gel electrophoresis and purified with Spin-X centrifuge tube filters (Trace Biosciences, Castle Hill, New South Wales, Australia). Products were cloned into the pGEM-T Easy vector (Promega Corporation, Madison, WI) and subjected to blue-white colony selection as instructed by the supplier. Automated sequencing was performed by the Automated DNA Sequencing Service, Sydney University Prince Alfred Macromolecular Analysis Centre.

Primer extension. Primer extension was carried out as previously described (5). RNA from MEL and NIH 3T3 cells was primed with an oligonucleotide specific for *Bklf* exon 1a, 5'-CCATCGATGGCGGCAAGAAACCCAGGCC CGGT-3', and was extended with avian myeloblastosis virus reverse transcriptase (Promega).

Cell culture. MEL, NIH 3T3, and COS cells were cultured at 37°C and 5% CO₂ in Dulbecco's modified Eagle's medium (low glucose) supplemented with 10% (vol/vol) heat-inactivated fetal calf serum and 1% (vol/vol) penicillin, strep-

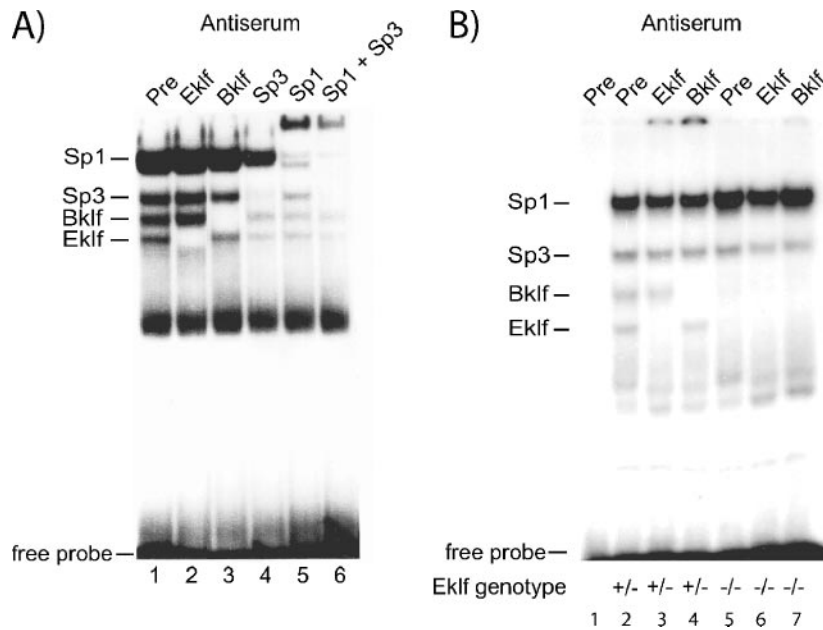


FIG. 1. Bklf protein is diminished in *Eklf*-null murine fetal liver. (A) EMSA showing the identity of proteins found in erythroid cells which recognize the β -major globin promoter CACCC box. MEL cell nuclear extracts were allowed to bind to a radiolabeled probe containing the β -major globin promoter CACCC box in the presence of either preimmune serum or an antibody specific for Eklf, Bklf, Sp1, and/or Sp3. (B) EMSA analysis of murine fetal liver nuclear extracts prepared from E14.5 *Eklf*-null and heterozygous (denoted below each lane) littermates. Lane 1 contains the β -major globin CACCC probe alone. Bklf and Eklf bands were confirmed by antibody supershifting. All lanes that lack either Eklf- or Bklf-specific antibody (α Eklf or α Bklf) contain preimmune serum as a control.

tomycin, and glutamine solution (Gibco-BRL Life Technologies, Grand Island, NY). *Drosophila melanogaster* Schneider line 2 (SL-2) cells were grown at 24°C in Schneider medium (Gibco) supplemented with 10% (vol/vol) heat-inactivated fetal calf serum and 1% (vol/vol) penicillin, streptomycin, and glutamine solution. B1.6 erythroblast cells were cultured as described previously (17). Cells were induced by the addition of 1 mM 4-hydroxytamoxifen (in ethanol) to a final concentration of 100 nM. As a negative control, ethanol was added to a final concentration of 0.0001% (vol/vol). RNA was extracted as described above at 48 h postinduction or at 0, 2, 4, 8, and 24 h postinduction for the time course study. For translation inhibition analyses, cycloheximide (made up in ethanol) was added to subconfluent cells to a final concentration of 5 μ g/ml. Thirty minutes later, cells were induced with 4-hydroxytamoxifen or with ethanol as a negative control as described above. RNA was extracted 8 h after tamoxifen induction to avoid cell death due to cycloheximide cytotoxicity.

ChIP. Chromatin immunoprecipitation (ChIP) assays were conducted with α Eklf and preimmune serum as previously reported (33, 34, 49). Immunoprecipitated DNA was analyzed by real-time PCR using the following primer sets: *Bklf* promoter 1a, 5'-GCTGCTCCACCCAAGTG-3' and 5'-ACCAACTCTGGCAGTACAT-3'; *Bklf* promoter 1b, 5'-CTGGGTGTGGCAGAATCTT-3' and 5'-GCCAGGGCGAGTCCAACCT-3'; negative control region approximately 10 kb upstream of *Bklf*, 5'-GCCTGCGGAGGTGATTAC-3' and 5'-TTCTGAAGCAAAGCCAAGAATATC-3'. β -major globin and *nectin* control primers have been described previously (33).

Vectors and cloning of the *Bklf* promoters. To clone *Bklf* promoter 1a, a 129SVJ mouse genomic library in Lambda FIX II vector (Stratagene, La Jolla, CA) was screened as suggested by the supplier. An approximately 500-bp sequence encompassing a portion of intron 1 and of exon 2 of *Bklf* was radiolabeled with [α -³²P]dCTP (1.7 \times 10⁶ cpm/ml) and was used to probe the library. A 2.3-kb NotI/NotI fragment containing exon 1a plus approximately 1.8 kb of upstream sequence and 400 bp of intron 1 was obtained from a positively identified clone and then subcloned into pBlueScript KS II⁻ (Stratagene) to create pBKS-Bklfprom1a. Two fragments (-189 to +89 and -532 to +89) were amplified from pBKS-Bklfprom1a by PCR with the forward primers 5'-ATTAGGTACCGGTCCAGCC TCTACTTACCC-3' and 5'-ATTAGGTACCGGAGCGCTCAGGCG-3', respectively, and the reverse primer 5'-ATTAAAGCTTAAACCCAGGCCCGGCTC-3'. The fragments were then subcloned into KpnI/HindIII pGL4.10[luc2] (Promega), a promoterless vector, to create pGL4.10[luc2]-Bklfprom1a(-189+89) and pGL4.10[luc2]-Bklfprom1a(-532+89). To clone *Bklf* promoter 1b, 286-bp (-225 to +61), 190-bp

(-129 to +61), 182-bp (-121 to +61) and 96-bp (-35 to +61) fragments were amplified from C57BL/6 genomic DNA (supplied by Richard Pearson, School of Molecular and Microbial Biosciences, Sydney, Australia) by PCR using the forward primers 5'-ATTAGGTACCTGGAAAGCTGGAGAGAGTC-3', 5'-ATTAGGTACCGGGTG TGGGCAGAATC-3', 5'-ATTAGGTACCGCAGAATCTTATCTGAAGCTATG-3', and 5'-ATTAGGTACCGCAGAAAAGTAAAATTGGGTG-3', respectively, and the reverse primer 5'-ATTAAAGCTTTTCATTGCTTGCTTCAGATG-3'. The fragments were subcloned into KpnI/HindIII pGL4.10[luc2] to form pGL4.10[luc2]-Bklfprom1b(-225+61), pGL4.10[luc2]-Bklfprom1b(-129+61), pGL4.10[luc2]-Bklfprom1b(-121+61), and pGL4.10[luc2]-Bklfprom1b(-35+61). pPac and pPac-Eklf were kindly provided by Menie Merika and Stuart Orkin (Harvard Medical School, Boston, MA). pSG5-Eklf was generously supplied by James Bieker (Mount Sinai School of Medicine, New York, NY).

Transactivation assays. SL-2 cells were transfected in six-well plates using the calcium phosphate method (72). Zero, 10, 50 or 100 ng pPac-Eklf (supplemented to a total 100-ng vector with pPac) was transfected along with 1 μ g pGL4.10[luc2], pGL4.10[luc2]-Bklfprom1a(-189+89), pGL4.10[luc2]-Bklfprom1a(-532+89), pGL4.10[luc2]-Bklfprom1b(-225+61), pGL4.10[luc2]-Bklfprom1b(-129+61), pGL4.10[luc2]-Bklfprom1b(-121+61), or pGL4.10[luc2]-Bklfprom1b(-35+61). For all transfections, 10 ng pGL4.74[*hRluc*/TK] (Promega) was included as a transfection control. Forty-eight hours posttransfection, cells were lysed and assayed for luciferase activity by using a dual-luciferase reporter assay system (Promega). MEL cells were transfected in six-well plates with 1 μ g of the pGL4.10[luc2] constructs and 10 ng pGL4.74[*hRluc*/TK] and were harvested 48 h later. Transfection was achieved with Tfx-50 reagent (Promega) per the manufacturer's instructions. In all cases, reporter activity was normalized with respect to *Renilla* luciferase levels.

Overexpression of Eklf in COS cells. Plates (100 mm) of COS cells, at 50% confluence, were transfected with 5 μ g pSG5-Eklf (or 5 μ g pMT3 as a negative control) using FuGENE6 (Roche Diagnostics) as instructed by the manufacturer. The medium was changed 48 h following transfection, and cells were harvested for nuclear extracts 24 h later as described previously (20).

Bioinformatics. GenBank cDNA and genomic searches were conducted using the BLAST algorithm (4) at the NCBI website (<http://www.ncbi.nlm.nih.gov/BLAST>). Full-length *Bklf* cDNAs from the DDBJ and GenBank were obtained from nucleotide searches (August 2006) at the NCBI website (<http://www.ncbi.nlm.nih.gov>). Additional start points of transcription were identified (August 2006) from oligonucleotide-cap cDNA entries at the Database of Transcriptional

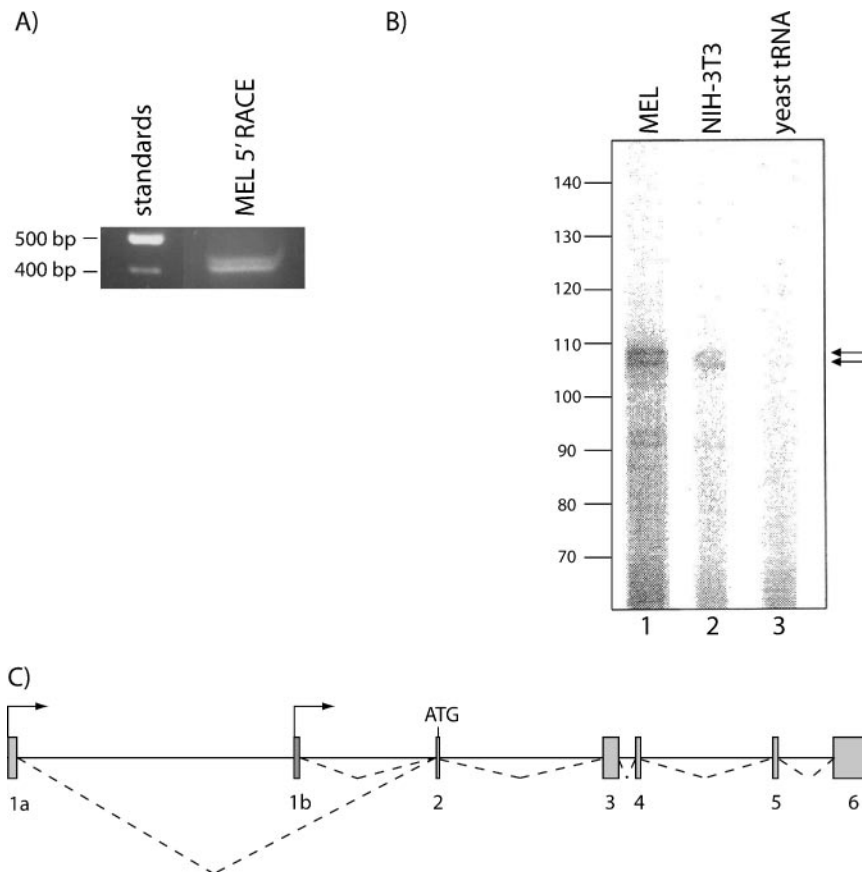


FIG. 2. Characterization of the transcriptional start sites of the alternative exons of *Bklf*. (A) 5' RACE from MEL cDNA generates two distinct bands which represent transcripts containing different lengths of the novel *Bklf* exon 1b. (B) Primer extension of *Bklf* mRNA using a primer specific for *Bklf* exon 1a. Lanes 1 and 2 contain primer-extended total RNA from MEL cells and NIH 3T3 cells, respectively. Lane 3 contains yeast tRNA as a negative control. Arrows indicate the two major start points of transcription for *Bklf* exon 1a. (C) A schematic of the murine *Bklf* locus showing the relative positions of the two alternative first exons, 1a and 1b. Exons are denoted by gray boxes. The ATG start codon is also shown.

Start Sites (DBTSS; <http://dbtss.hgc.jp/>). Vertebrate conservation analyses were performed with the University of California—Santa Cruz (UCSC) Genome Browser (February 2006; <http://genome.ucsc.edu/>) using the mouse (mm8) genome assembly (44).

Nucleotide sequence accession number. A full-length *Bklf* cDNA entry containing exon 1b has been submitted to GenBank (accession number DQ981866).

RESULTS

***Bklf* mRNA is reduced in *Eklf*-null cells.** Several studies have employed a probe containing the critical CACCC box of the β -major globin promoter in EMSAs to elucidate DNA-binding proteins in erythroid cells (20, 32). The major CACCC-binding activities detected include those of Sp1, Sp3, Eklf, and Bklf (Fig. 1A). While the antibodies for Sp1 and Sp3 produce some nonspecific inhibition of binding of other proteins, the annotation of Sp1 and Sp3 in Fig. 1A is consistent with previous identifications of these prevalent CACCC-binding factors (12, 31). Interestingly, when E14.5 *Eklf*^{-/-} fetal liver extracts are examined, the DNA-binding activity of Bklf, but not that of Sp1 or Sp3, is reduced (Fig. 1B). This result is in accordance with previous observations, including Western blot experiments, that show that Bklf protein levels are diminished in *Eklf*-null fetal liver but not in nonerythroid tissues (20). These results raise the possibility that Bklf is directly or indirectly

dependent on Eklf for its expression, stability, or activity in erythroid cells.

Eklf is known to be a potent activator of transcription. We first investigated whether Bklf's dependence on Eklf is observed at the mRNA level. RNA from E14.5 livers of *Eklf*^{-/-}, *Eklf*^{+/-}, and wild-type murine fetuses was analyzed by real-time RT-PCR using *Bklf* specific primers. *Bklf* transcripts were underrepresented in *Eklf*^{-/-} fetal livers compared to the fetal livers of *Eklf*^{+/-} and wild-type littermates (see Fig. 5C). In accordance with this result, a recent microarray study on genes that are differentially expressed in *Eklf*^{-/-} fetal liver also identified *Bklf* as such a downregulated gene, although this result was not further validated (33). In addition, *Bklf* mRNA is diminished in *Eklf*^{+/-} compared to wild-type fetal liver, suggestive of dose dependency. Taken together, these data suggest that Eklf is required for the direct or indirect activation of the *Bklf* gene at the transcriptional level.

Identification of a novel *Bklf* leader exon, 1b. *Bklf*'s expression is highly dependent on Eklf in erythroid tissue, yet *Bklf* mRNA is also expressed in other tissues, for instance, the fetal head and the adult lung, where Eklf is not abundant (20). Many genes have multiple promoters that allow their expression in a variety of different biological contexts (6). We hy-

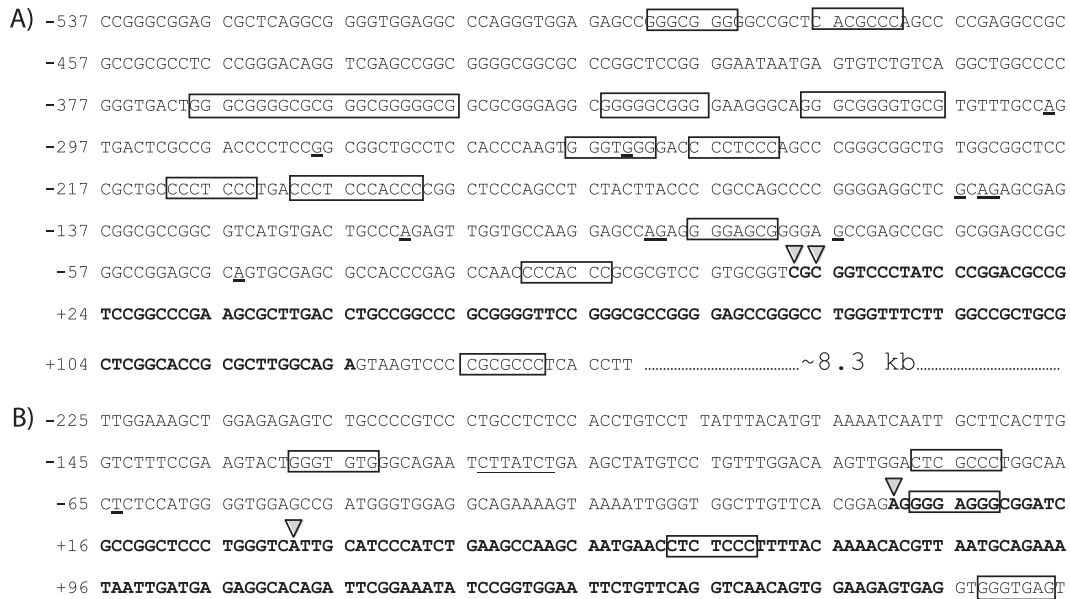


FIG. 3. The sequence of *Bklf* exon 1a (A) and exon 1b (B) and their flanking genomic regions. Note that approximately 8.3 kb of intron 1 has been omitted. Exons are written in boldface type, and their transcriptional start sites, as determined by results shown in Fig. 2A and B, are denoted by triangles. For each exon, the 5'-most position of these start sites has been designated position +1. For exon 1b, an upstream transcriptional start site that was infrequently detected by 5' RACE cloning is indicated with a boldface underline. For exon 1a, other putative transcription start sites are underlined. These transcription start sites were inferred from full-length cDNA entries in GenBank (DDBJ accession numbers AK007959 [42], AK157576, AK143838, and AK010713 [14, 41]; GenBank accession numbers BC116938 and BC119214 [77]) and from queries of the DBTSS (clone names BY338164, BY724103, BY707959, BY742654, BY732683, BY732370, BY188922, BY062971, and BB628801 [45, 80]). Motifs that precisely fit the Eklf binding consensus of 5'-NCNCNCCC-3' (or its reverse complement) are boxed. The putative Gata-binding site in promoter 1b is underlined.

pothesized that *Bklf* may utilize several promoters to achieve its broad expression profile.

To explore this possibility, we used 5' RACE with a primer targeted to exon 3 of *Bklf*. Two distinct fragments were amplified from MEL cell RNA (Fig. 2A) and were subsequently cloned into pGEM-T Easy vector and sequenced. The two fragments were identical, with the exception that one contained an additional 31 base pairs at its 5' end (Fig. 3). As anticipated, this fragment contained a portion of exon 3 (up to the RACE primer) and exon 2, but it also contained an alternative first exon which differed from the previously published leader exon of *Bklf* (RefSeq accession number NM_008453; 42) (Fig. 3). A BLAST analysis of the mouse genome revealed that this novel first exon, hereby named exon 1b, lies within the first intron of *Bklf* (Fig. 2C). 3' RACE confirmed that transcripts containing exon 1b extend to include all the exons of the gene (data not shown). A USCS Genome Browser analysis of the putative promoter region immediately upstream of this novel exon revealed a high level of conservation across a wide range of divergent vertebrates including the chicken (*Gallus gallus*) and the frog (*Xenopus tropicalis*) (data not shown). This result supports the view that the novel promoter (1b) is of functional importance. The two identified promoters (1a and 1b) generate transcripts that contain a single variant leader exon (exon 1a or 1b) and five common downstream exons. The AUG start codon lies within exon 2; hence, both transcripts encode the same Bklf protein. The sequence of this novel *Bklf* cDNA was submitted to GenBank.

We carried out additional experiments to further define the

start points of transcription. 5' RACE investigations using a number of *Bklf*-specific primers consistently yielded fragments exhibiting the two exon 1b putative transcription start sites shown in Fig. 3 (data not shown). A single clone was identified, however, which contained an additional 64 base pairs of non-coding 5' sequence, suggestive of a weak upstream transcriptional start site (Fig. 3).

Despite the frequent detection of 5' RACE products containing exon 1b, no products were found to contain the previously identified first exon (1a), which is present in the majority of *Bklf* cDNAs lodged at the DDBJ and GenBank. Their absence is possibly due to the high GC content (approximately 77%) of exon 1a compared to that of exon 1b (approximately 46%), which the RACE polymerase may have difficulty reading through. Therefore, we employed primer extension, using a primer specific for exon 1a, to determine the start sites of transcription for this upstream promoter. In this way, two major transcription start sites were identified (Fig. 2B and Fig. 3). An analysis of cDNA entries lodged at DDBJ, GenBank, and the DBTSS revealed multiple other proximal start sites (Fig. 3). This result suggests that promoter 1a is a typical GC-rich promoter that exhibits numerous start sites of transcription.

***Bklf* promoter 1b is active in erythroid tissues.** To investigate whether the two identified *Bklf* promoters have distinct expression patterns, we examined the levels of mRNA they produced in a range of adult murine tissues. Real-time RT-PCR primers were designed to amplify regions crossing the exon 1a/2 and exon 1b/2 boundaries, respectively. This allowed discrimination between the two different transcripts and also

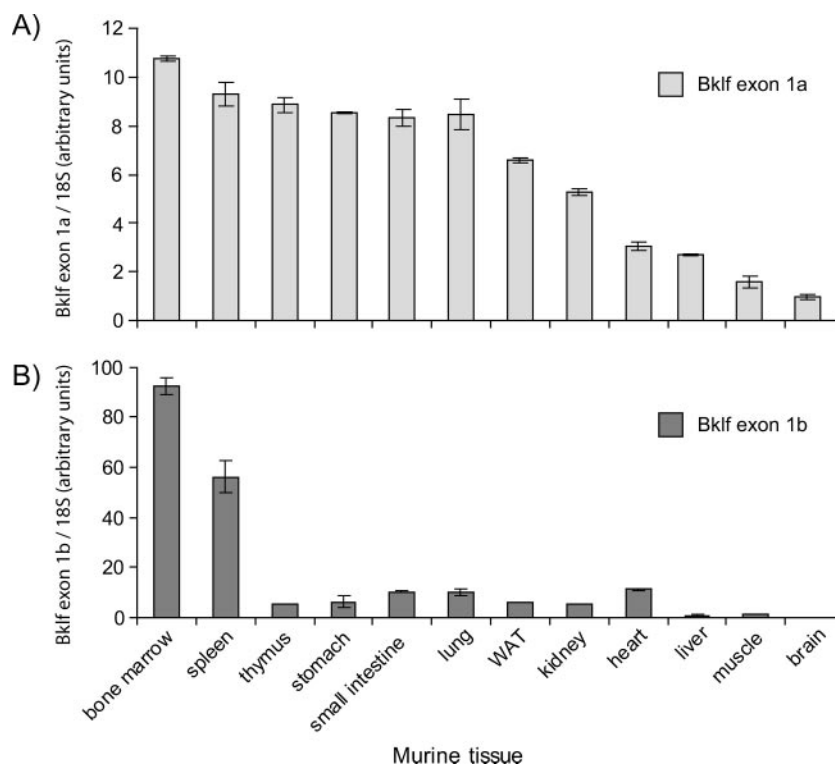


FIG. 4. The downstream *Bklf* promoter is active predominantly in hematopoietic tissue. The levels of the two *Bklf* transcripts in murine tissues were determined by quantitative real-time RT-PCR. (A) Levels of *Bklf* transcripts generated from the upstream promoter and containing exon 1a. (B) Levels of *Bklf* mRNA arising from the downstream promoter and containing exon 1b. All values were normalized to 18S rRNA levels. WAT, white adipose tissue.

reduced the amplification of any trace amounts of contaminating genomic DNA. Transcripts from promoter 1a were found ubiquitously (Fig. 4A). In contrast, transcripts containing exon 1b were found at high levels in the bone marrow and spleen (Fig. 4B). Interestingly, *Eklf*'s expression is restricted to these two hematopoietic organs (56). This result raised the possibility that promoter 1b might be dependent on *Eklf* and may account for the reduction in *Bklf* mRNA observed with the *Eklf*^{-/-} fetal livers. Quantitative assays using known amounts of amplicon as template suggest that levels of the two *Bklf* transcripts are roughly equivalent in erythroid tissue (data not shown).

***Bklf* promoters 1a and 1b are dependent on *Eklf* in erythroid cells.** Given the restricted expression of transcripts containing exon 1b (Fig. 4B), we reasoned that promoter 1b might be highly dependent on *Eklf* and that its inactivity might contribute to the reduction in *Bklf* protein levels in the *Eklf*^{-/-} fetal liver. We found exon 1b transcripts at approximately 30-fold lower levels in *Eklf*^{-/-} than in wild-type fetal livers (Fig. 5B). Interestingly, transcripts containing exon 1a that arise from the ubiquitous promoter were found at approximately 10-fold lower levels in the null fetal livers (Fig. 5A). This suggests that both promoter 1b and, to a lesser extent, promoter 1a are activated by *Eklf*. It should be noted, however, that *Eklf* is clearly not crucial for expression from promoter 1a, as evidenced by its ubiquitous activity (Fig. 4A).

***Eklf* activates both *Bklf* promoters in transactivation assays.** To further explore the possibility that *Eklf* can activate tran-

scription from both promoters, we first performed transactivation experiments with *Drosophila* SL-2 cells, which are often used to examine Klf proteins since they lack CACCC-binding activity compared to mammalian cells (18). Fragments of both *Bklf* promoters were cloned into the promoterless reporter construct pGL4.10[*luc2*]. *Bklf* promoter 1a is GC rich and contains many elements that fit the *Eklf*-binding consensus sequence of 5'-NCNCNCCCN-3' (Fig. 3). A 278-bp proximal promoter region from -189 to +89 that contains several canonical *Eklf* recognition sites was activated slightly in the presence of *Eklf* (Fig. 6A). An extended promoter element (-532 to +89) showed higher activation by *Eklf*, thus suggesting that CACCC boxes upstream of -189 contribute to *Eklf* activation (Fig. 6A). We also tested promoter 1b and found that the region -225 to +61 is potentially activated by *Eklf* (Fig. 6B). This region contains three canonical *Eklf* recognition sites. A truncated fragment (-129 to +61) which retains all of these CACCC sites is activated by *Eklf* to a similar degree (Fig. 6B). In particular, one of the potential *Eklf*-binding sites (-123 to -129) is identical to the critical β -major globin promoter CACCC box, which is bound by *Eklf* in vivo. Deleting this motif results in a >50% reduction in promoter activity in response to *Eklf* (Fig. 6B, compare the -129 to +61 construct with the -121 to +61 construct). In addition, EMSA analysis demonstrated that *Eklf* can bind this *Bklf* promoter 1b CACCC box (Fig. 6C). Lastly, a further truncated promoter construct (-35 to +61), which contains only one of the three canonical *Eklf*-binding sites, was found not to be activated by

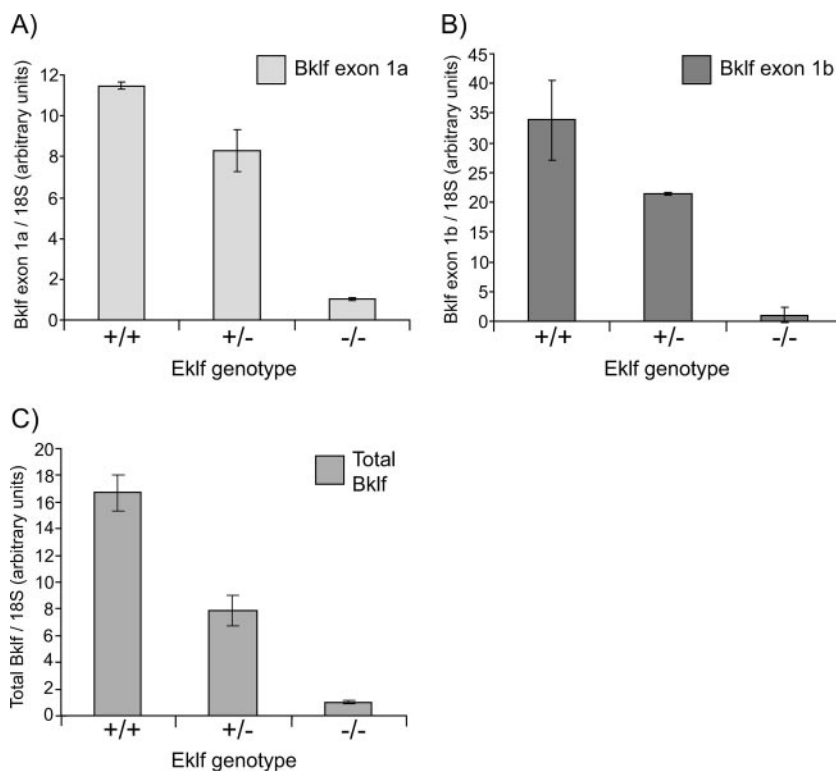


FIG. 5. Both *Bklf* promoters are less active in E14.5 *Eklf*^{-/-} murine fetal liver. Analysis of the two *Bklf* transcripts in E14.5 littermate *Eklf*^{+/+}, *Eklf*^{+/-}, and *Eklf*^{-/-} fetal livers was performed by quantitative real-time RT-PCR. (A) *Bklf* exon 1a transcripts. (B) *Bklf* exon 1b transcripts. (C) Total *Bklf* transcripts: *Bklf*-specific primers were designed to amplify a region spanning the exon 4/5 boundary. *Bklf* mRNA levels were normalized to 18S rRNA levels.

Eklf (Fig. 6B). This implies that the 3'-most CACCC box (+3 to +9) is not sufficient for *Eklf* activation of *Bklf* promoter 1b. In addition, all promoter 1a and 1b constructs that displayed *Eklf*-mediated activation in SL-2 cells were also active in MEL cells (data not shown); the MEL cell line expresses *Eklf* (Fig. 1A). Taken together, these results corroborate the data presented here that both *Bklf* promoters, particularly promoter 1b, are responsive to *Eklf*.

***Eklf*-ER activates the endogenous *Bklf* promoter 1b in B1.6 cells.** To investigate the dependence of *Bklf* on *Eklf* in a more physiological system, the B1.6 cell line was used. The B1.6 line is an erythroid cell line derived from *Eklf*-null knockout mice into which a transgene encoding a tamoxifen-inducible *Eklf*-ER protein has been introduced (17, 50). Real-time RT-PCR analysis demonstrated that, prior to activation of *Eklf*-ER by tamoxifen, transcripts from promoter 1a were undetectable, while mRNA from promoter 1b was at a trace level (Fig. 7A). Following induction, transcripts from promoter 1a remained undetected while promoter 1b was activated strongly. cDNA levels of readily detectable *Klfs*, such as *Klf13* and *Klf16*, which were used as controls, displayed no change upon induction. This result further provides strong evidence that promoter 1b is activated, either directly or indirectly, by *Eklf*. Promoter 1a, on the other hand, appears to be nonresponsive to *Eklf*-ER in this cellular system.

We also examined other *Klfs*. Interestingly, *Klf10* showed an approximately eightfold increase in expression upon induction (Fig. 7A). This result corroborates a previous microarray ob-

servation that *Klf10* levels increase upon *Eklf*-ER induction (33). *Klf10* transcripts were also found to be reduced approximately fourfold in *Eklf*-null compared to wild-type fetal liver (Fig. 7B). This result further suggests that *Klf10* is also regulated by *Eklf* in erythroid cells, although not to the extent that is observed for *Bklf* (compare Fig. 5).

***Eklf*-ER activates *Bklf* promoter 1b in the presence of cycloheximide.** It was possible that *Eklf*-ER was indirectly activating *Bklf* via some intermediate downstream targets. We investigated this possibility by exposing B1.6 cells to cycloheximide, a translation inhibitor, prior to and during induction with tamoxifen. Due to the cytotoxicity of cycloheximide, cells were harvested 8 h (rather than 48 h) after induction. Activation of promoter 1b occurred in induced cells in the presence of cycloheximide, suggesting that the *Bklf* gene is a direct target of *Eklf* (Fig. 8A). In addition, *Klf10* mRNA was also upregulated by *Eklf*-ER in the presence of cycloheximide, although to a lesser degree than *Bklf*, thus suggesting that it too is a direct target of *Eklf* (Fig. 8B).

We also performed a time course study of *Bklf* and *Klf10* mRNA levels following induction. It was expected that indirect target genes would exhibit a considerable lag phase before being expressed. However, statistically significant increases in *Bklf* exon 1b and *Klf10* transcripts were detected after only 2 and 4 h postinduction, respectively, thus providing further evidence that the *Bklf* and *Klf10* genes are direct targets of *Eklf* (Fig. 8C and D).

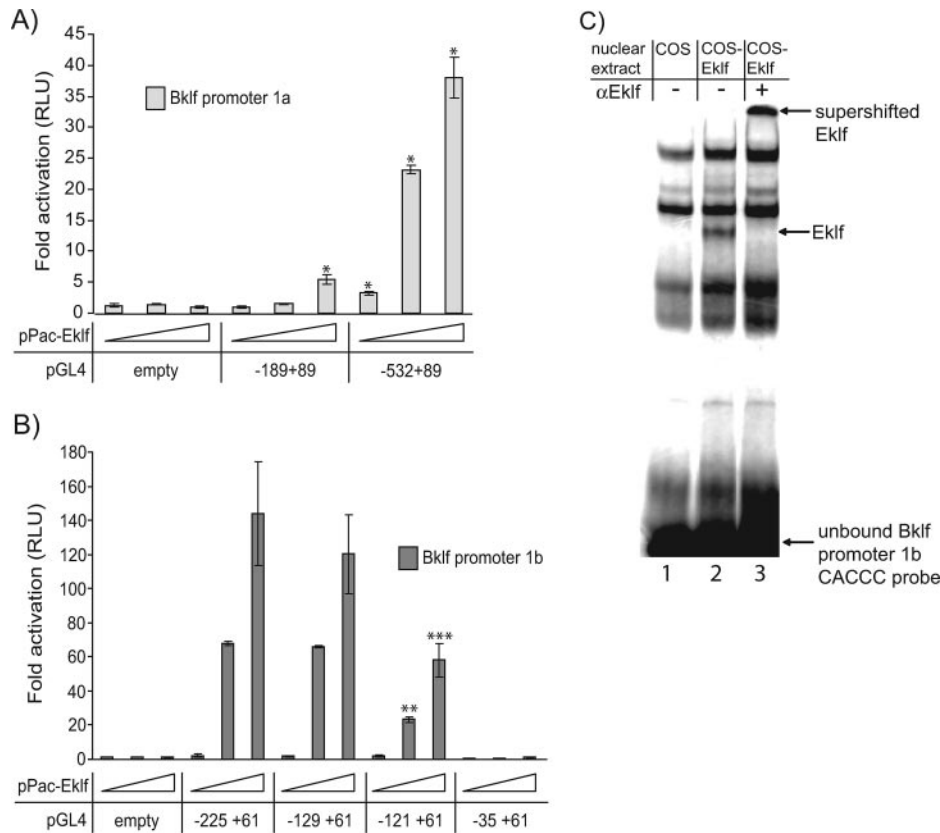


FIG. 6. Eklf transactivation assays of the *Bklf* promoters in SL-2 cells. Zero, 10, 50, or 100 ng pPac-Eklf (supplemented to 100 ng with pPac) was cotransfected with 1 μ g pGL4.10[*luc2*] reporter vector containing fragments of *Bklf* promoter 1a (–189 to +89 or –532 to +89) (A) or varied lengths (–225 to +61, –129 to +61, –121 to +61, or –35 to +61) of *Bklf* promoter 1b (B). Firefly luciferase activity has been expressed as activation above that observed for cells transfected with each reporter vector and pPac alone. (A and B) Cell lysates were assayed for firefly luciferase activity 48 h posttransfection. Reporter activity has been normalized to *Renilla* luciferase levels. *, $P < 0.05$ compared to pGL4.10[*luc2*]-empty cotransfected with 10, 50, or 100 ng pPac-Eklf as appropriate (paired Student's *t* tests). **, $P < 0.0005$ and ***, $P < 0.05$ compared to pGL4.10[*luc2*]-*Bklf*prom1b(–129+61) cotransfected with 50 ng and 100 ng pPac-Eklf, respectively (paired Student's *t* tests). (C) EMSA analysis demonstrating that Eklf binds in vitro a CACCC box present in *Bklf* promoter 1b. Nuclear extracts were allowed to bind to a radiolabeled probe containing the *Bklf* promoter 1b CACCC box in the presence of either anti-Eklf (lane 3) or preimmune serum (lanes 1 and 2). RLU, relative light units.

Eklf-ER binds endogenous *Bklf* promoters 1a and 1b in B1.6 cells. To confirm that Eklf activates *Bklf* by direct binding, we conducted ChIP assays using α Eklf antiserum to detect the presence or absence of Eklf-ER at the *Bklf* promoters. ChIP

material was obtained from uninduced and tamoxifen-induced B1.6 cells. As a positive control, Eklf-ER was detected at the β -major globin promoter, a known target of Eklf (Fig. 9E). Upon induction of the cells, Eklf-ER was found to associate

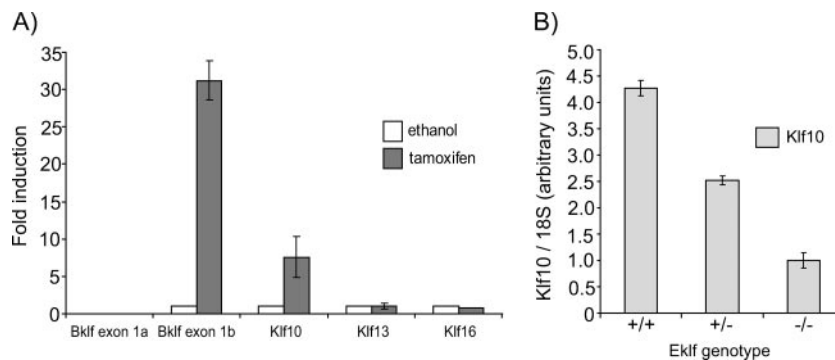


FIG. 7. *Bklf* exon 1b and *Klf10* transcripts are upregulated in the presence of Eklf. (A) *Klf* mRNA levels in B1.6 cells that were harvested 48 h following induction with either 100 nM tamoxifen or with ethanol (0.0001%) as a control. (B) *Klf10* transcript levels in *Eklf*^{+/+}, *Eklf*^{+/-}, and *Eklf*^{-/-} fetal livers. All mRNA levels were determined by quantitative real-time RT-PCR and normalized to 18S rRNA levels.

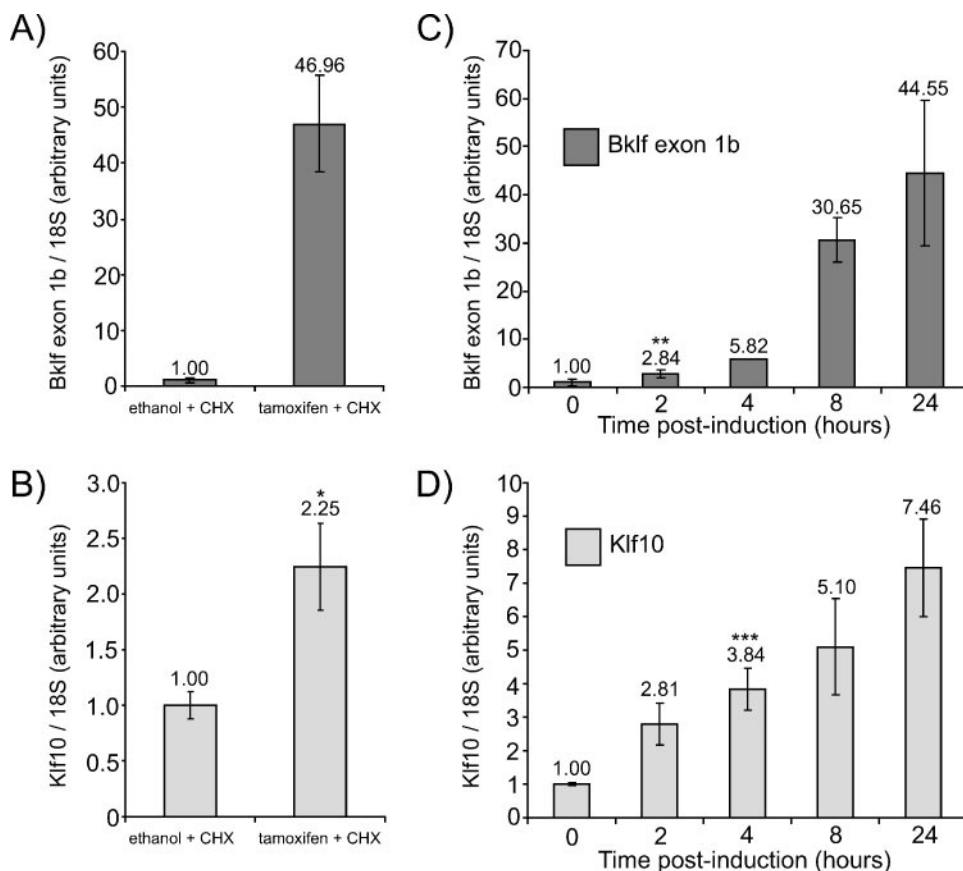


FIG. 8. *Bklf* and *Klf10* are directly activated by Eklf-ER. (A and B) B1.6 cells were exposed to cycloheximide (CHX) 30 min before induction with tamoxifen and were harvested for RNA 8 h thereafter. *Bklf* exon 1b levels (A) and *Klf10* mRNA levels (B) were determined by quantitative real-time RT-PCR. (C and D) B1.6 cells were harvested at numerous time points after tamoxifen induction in the absence of cycloheximide. Again, *Bklf* exon 1b levels (C) and *Klf10* mRNA levels (D) were determined by quantitative real-time RT-PCR. In all cases, 18S rRNA levels were used for normalization. *, **, and ***, $P < 0.05$ (paired Student's t test, compared to ethanol control or 0-h time point as appropriate).

with *Bklf* promoter 1b. No Eklf-ER was detected in negative control regions: the *nectin* gene and a region 10 kb upstream of the *Bklf* locus (Fig. 9B to D). Interestingly, Eklf-ER was also detected at *Bklf* promoter 1a following induction of the cells (Fig. 9A). This was somewhat unexpected given that no *Bklf* transcripts from promoter 1a were observed in induced B1.6s (Fig. 9A). Nonetheless, it is consistent with the previous observation that transcripts containing *Bklf* exon 1a are reduced in *Eklf*-null fetal liver (Fig. 5A).

DISCUSSION

Many erythroid genes utilize alternative promoters to achieve complex expression patterns (81). In particular, several genes involved in the heme biosynthesis pathway, including *ALAD*, *PBGD*, and *UROS*, possess an upstream housekeeping promoter that enables widespread expression and a downstream promoter that is active in erythroid cells (2, 3, 16, 43). Similarly, the genes encoding the transcription factors *p45 Nf-e2* and *Gata-2* each have dual promoters with housekeeping and erythroid roles (57, 59, 65). We have shown here that murine *Bklf* is another erythroid gene that is regulated by at least two promoters that exhibit different expression profiles but produce transcripts encoding identical proteins.

Transcripts containing exon 1a were readily detected by RT-PCR in all tissues examined, thus suggesting that the upstream promoter (1a) is widely active. In this sense, it can be considered a housekeeping promoter that allows *Bklf* expression in both erythroid and nonerythroid cells and tissues. Promoter 1a is GC rich, a trait which is characteristic of promoters which generate ubiquitous transcripts (15, 73, 93). In addition, oligonucleotide-cap cDNA analysis reveals that promoter 1a has multiple transcriptional start sites spread over a region of approximately 300 bp. It is well established that most genes exhibit some fluctuation in their start points of transcription (79). However, this phenomenon has been noted to be particularly pronounced for GC-rich, housekeeping promoters that lack a TATA box, as is the case for *Bklf* promoter 1a (13, 15).

In contrast, the novel downstream promoter 1b is active primarily in hematopoietic tissues, such as the fetal liver, adult spleen, and bone marrow and in erythroid cells, such as the tamoxifen-induced B1.6 erythroblast line and MEL cells. In contrast to transcripts containing exon 1a, mRNA containing exon 1b was barely detected in tissues such as the liver and skeletal muscle and was undetected in the brain. This result suggests that, unlike promoter 1a, promoter 1b does not exhibit a widespread basal level of activity. Consistent with its

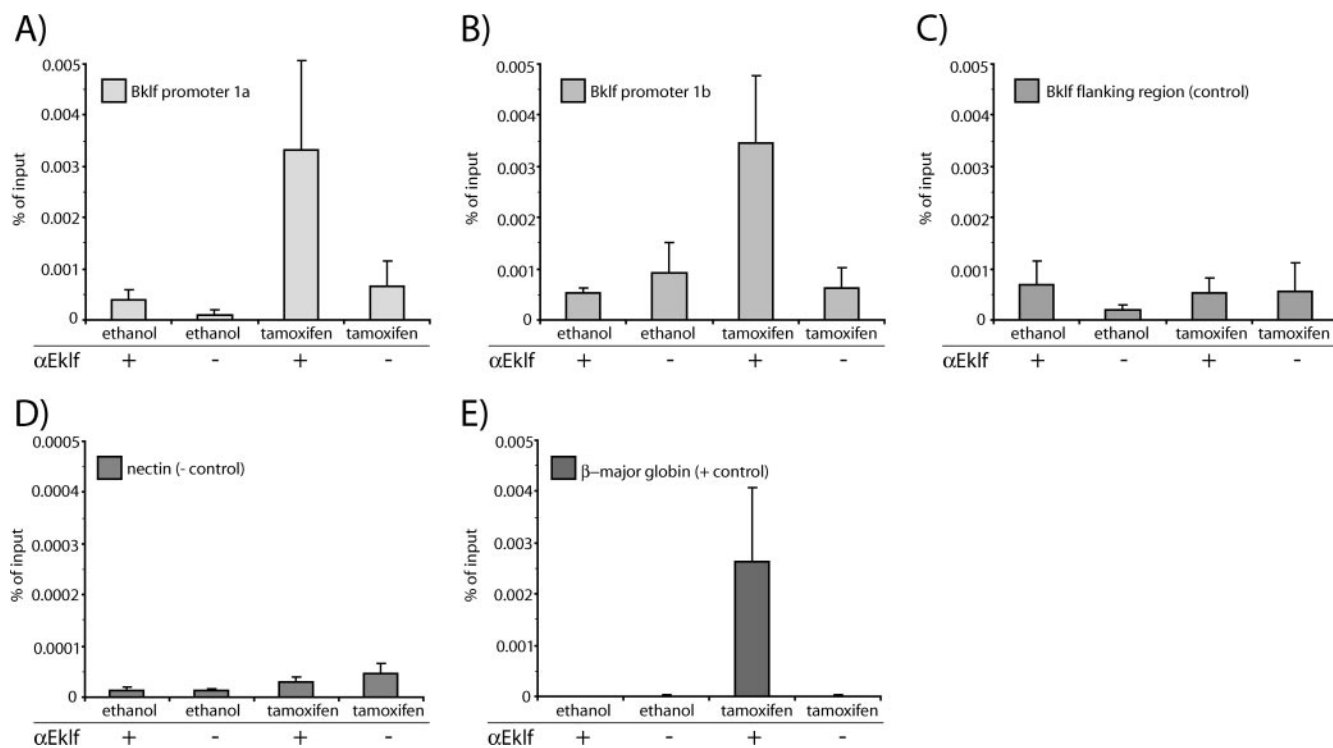


FIG. 9. Eklf ChIP at the two *Bklf* promoters. ChIP material was extracted from B1.6 cells that were induced with tamoxifen or ethanol (control). Chromatin was immunoprecipitated using an Eklf antibody (denoted by "+") or preimmune serum (-). Primers used for real-time PCR quantification were targeted against *Bklf* promoter 1a (A), *Bklf* promoter 1b (B), a control region 10 kb upstream of the *Bklf* locus (C), the *nectin* promoter (D), and the β -major globin promoter (E).

restricted activity, promoter 1b is noticeably less GC rich than promoter 1a and does not resemble a typical housekeeping promoter. It lacks a canonical TATA box, although it does have an AT-rich element (TAAAT) at -26 to -21. In addition, promoter 1b contains a motif that perfectly fits the consensus of a downstream promoter element (+28 A/G G A/T C/T G/A/C +32) (47).

In concordance with its erythroid cell-restricted activity, we have demonstrated here that *Bklf* promoter 1b is directly activated by Eklf-ER in the B1.6 erythroid cell line. It is also highly dependent on Eklf in murine fetal liver. This dependence presumably accounts for the expression of transcripts containing exon 1b being restricted to hematopoietic tissues, such as the spleen and bone marrow, where Eklf is known to be present (56). A 190-bp region of promoter 1b (-129 to +61) is strongly activated by Eklf in reporter assays with SL-2 cells. This region contains three elements that fit the Eklf-binding consensus. One of these elements (centered around -126) is identical to the proximal CACCC box of the β -major globin promoter (32), albeit on the opposite strand, and is bound by *Eklf* in vitro. Deletion of this CACCC box leads to a significant reduction in the activation of the promoter by Eklf. Interestingly, in *Bklf* promoter 1b, this CACCC box is flanked by a motif that fits the Gata-binding consensus of 5'-A/T GATA A/G-3' (25, 54, 89). Gata-1 has previously been noted to physically interact with Eklf and to synergistically potentiate transcriptional activation (55).

The ubiquitously active promoter 1a also appears to be dependent on Eklf in erythroid cells. Exon 1a transcripts are

markedly reduced in the *Eklf*^{-/-} fetal liver. In B1.6 cells, Eklf-ER is detected at promoter 1a by ChIP analysis; however, no resultant transcripts in B1.6 cells are detected. It is possible that other factors that are required for promoter 1a activity are absent in the cells, perhaps as a side effect of the immortalization process. While the -189 to +89 fragment of promoter 1a displays only mild activation by Eklf in SL-2 cells, an extended region (-532 to +89) is strongly activated. This result suggests that CACCC boxes between -532 and -189 contribute significantly to Eklf activation of *Bklf* promoter 1a.

Despite its dependence on Eklf in erythroid cells, *Bklf* promoter 1a is also comparably active in nonerythroid tissues where Eklf is absent, such as lung, gastrointestinal, and white adipose tissues. This implies that in nonerythroid tissues, promoter 1a may be bound by factors which are capable of substituting for Eklf and which are sufficient to activate transcription. Indeed, it is possible that other members of the Klf family may utilize Eklf's cognate binding site(s) in promoter 1a and thus drive *Bklf* expression in nonerythroid tissues. Within the family, lung Krüppel-like factor (Lklf/Klf2) and gut-enriched Krüppel-like factor (Gklf/Klf4) are the most highly related to Eklf (9, 21, 38, 78, 85) and, hence, these two factors are suitable candidates to assume such a compensatory role. This hypothesis is interesting and raises the possibility that promoter 1a and, by inference, other typical GC-rich housekeeping promoters, may be ubiquitously expressed by virtue of being dependent on some tissue-restricted Klf in some tissues and other Klf in other tissues, rather than being dependent exclusively on ubiquitous GC-box-binding proteins, such as

Sp1. However, if compensation by other Klf is occurring, the question of why promoter 1b is not similarly active in non-erythroid tissues is raised. A possible explanation for this is that promoter 1b may contain sequence elements other than its Eklf-binding site(s) which are bound exclusively by erythroid factors which, in turn, are crucial for transcriptional activation, for instance, Gata-1.

Eklf has been found to function predominantly as an activator, whereas Bklf is generally a repressor. Both proteins have also been shown to bind *in vitro* to an overlapping set of CACCC elements found in erythroid promoters, such as those of the β -globin, carbonic anhydrase I, Gata-1, Pbgd, and pyruvate kinase genes as well as in globin locus control region DNase hypersensitive sites (20). Therefore, given the opposing roles of the proteins in transcriptional regulation, the most likely consequence of Eklf's activation of *Bklf* is that it serves to temper Eklf's stimulatory effects. That is to say that Bklf upregulation inhibits the unbridled activation of Eklf's target genes. In support of this idea, reporter assays conducted with SL-2 cells have shown that Bklf can silence Eklf activation of a CACCC-containing promoter (84). There is also evidence that *Bklf* promoter 1b is itself subject to autorepression. In B1.6 cells, *Bklf* exon 1b levels are higher in the cycloheximide-treated cells than in untreated cells (compare Fig. 8A and C at the 8-h time point). This is one possible consequence of unchecked Eklf-mediated activation.

Such transcriptional antagonism has been observed among members of the Klf family in reporter assays using a number of promoters, including those of the *Ap-2 α* (36), *cytochrome p4501A1* (*Cyp1a1*) (35, 39, 40, 95), and *cyclin D1* (48, 60, 74, 96) genes. In particular, Klf4 and Klf5 have been shown to exhibit opposing effects at a number of promoters in transient transfection experiments. Klf4 represses while Klf5 activates the smooth muscle *SM α -actin* (51) and *SM22- α* (1) promoters as well as the *cyclin D1* (60, 74) and *laminin- α 1* (69) promoters. It is possible that, similar to Klf4 and Klf5, Eklf and Bklf may demonstrate contrasting functions in order to fine-tune the expression of a set of common target genes. Future studies will endeavor to identify target genes of Bklf which are either shared with Eklf or which are distinct, due to subtle differences in the binding preferences of the two transcription factors.

Further evidence of cross-regulation within the Klf family comes from the observation in this study that Eklf also upregulates *Klf10* expression in the fetal liver and in tamoxifen-treated B1.6 erythroblasts. Klf10 is a negative regulator of cell cycle progression (11, 37, 82). Its induction is therefore consistent with the reduced proliferation of B1.6 cells observed after Eklf-ER induction. Interestingly, the human *KLF10* gene contains two alternative promoters (26). The upstream promoter is widely active, while the function of the downstream promoter is unclear (26). It is possible that these alternative promoters are conserved in the mouse and that one or both may be responsive to Eklf.

The results presented here provide evidence that the Klf family is subject to internal hierarchical regulation, with Eklf activating the *Bklf* and *Klf10* genes in erythroid cells. Other examples of cross-regulation and autoregulation have also been inferred from transient transfection experiments and work with cell lines. Klf8 binds and represses the *Klf4* promoter (92). In addition, reporter assays have demonstrated that Klf4

is able to activate its own promoter, while Klf5 represses it (22). Further indirect evidence of hierarchical regulation comes from observations of concomitant changes in Klf expression during lymphocyte differentiation. For example, Klf2, Bklf, and Klf4 are expressed in resting B lymphocytes and are downregulated upon activation through the B-cell receptor (28, 30, 94). Similarly, Eklf, Klf2, and Bklf are expressed in quiescent T cells and are downregulated following activation (83, 94). Another member of the KLF family, KLF13, is also regulated during T-cell activation, albeit at a posttranscriptional level. As such, KLF13 protein, but not mRNA, is upregulated during T-cell activation (62, 76). Taken together with the results presented here, these studies allude to a complex web of hierarchical regulation which presumably functions to finely adjust the expression patterns of downstream target genes.

The widespread expression of Bklf, combined with potential redundancy by other members of the Klf family, has complicated interpretations of the *Bklf*-null mice data (not shown). However, although determination of the biological role of Bklf has been difficult, the results presented here provide strong *in vivo* evidence that Bklf is dependent on the erythroid transcriptional activator Eklf. Eklf has been shown to be crucial for erythropoiesis and directly binds to and regulates numerous erythroid promoters, including that of the adult β -globin gene. The observation that *Bklf* has two Eklf-dependent promoters, of which one appears to be active predominantly in erythroid tissues, strongly suggests that Bklf performs an erythroid role *in vivo*. Investigations on whether Bklf represses genes activated by Eklf *in vivo* are continuing.

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