Erythroid Kruppel-like Factor (EKLF) is an erythroid-specific transcription factor that plays a critical role in γ- to β-globin gene switching during development. To identify essential domains required for EKLF transactivation function, we cotransfected a human erythroleukemia cell line (K562) with a locus control region γ/Luc/β/Cat reporter and an EKLF expression vector. In this assay EKLF mediates a 500-fold induction of β/CAT expression compared with controls. To map essential transactivation domains, progressive NH2-terminal and internal deletion mutants of EKLF were constructed. All EKLF mutants were expressed at wild-type levels, localized to the nucleus, and bound DNA. When mutant EKLF proteins were tested for β/CAT activation, a novel transactivation domain was identified. This novel domain, encompassing amino acids (aa) 140–358, is sufficient for maximal β/CAT activation. An 85-amino acid subdomain within this region (aa 140–225) is essential for its activity. Interestingly, this central transactivation subdomain is functionally redundant with the amino-terminal domain (aa 1–139). Thus, EKLF possesses at least two potent transactivation domains that appear to function in a redundant manner.

The genes encoding β-like subunits of human hemoglobin are expressed in a tissue- and developmental stage-specific pattern of expression (1). Expression is exclusive to erythroid tissues, and ε-, γ-, and β-globin gene expression is predominately restricted to the yolk sac, fetal liver, and bone marrow, respectively. The genes are present within a 100-kb1 locus that contains a potent 22-kb enhancer termed the locus control region (LCR) (2–5). Several groups have proposed that precise tissue and developmental regulation is accomplished by complex protein-protein interactions between factors that bind the LCR and those that bind the promoters of individual genes (6, 7). The competition model suggests that a number of erythroid-specific and ubiquitous factors bind the LCR and enable this region to function as a potent enhancer. Downstream genes then compete for productive interactions with the LCR (2–5). Presumably, yolk sac-, fetal liver-, and bone marrow-specific factors bind to globin gene promoters and proximal enhancers and provide individual genes with a competitive advantage for interaction with the LCR at the appropriate developmental stage.

Erythroid Kruppel-like Factor (EKLF) is one factor that is critical for the developmental stage-specific switch from the γ- to β-globin expression (reviewed in Ref. 8). This erythroid-specific transcription factor binds the β-globin promoter and activates high level expression (9, 10). EKLF is a 358-amino acid protein containing an amino-terminal, proline-rich transactivation region (aa 1–275) and a COOH-terminal DNA binding domain (aa 276–358) (10). The DNA binding domain consists of three C2H2 Kruppel-like zinc fingers that bind specifically to the CCACACCT motif at −90 of the β-globin promoter (11). Although EKLF also binds to the CACCC box in the γ promoter, the binding affinity to γ CACCC is 8-fold lower than the binding affinity for β CACCC and EKLF preferentially activates the β gene in transient transfection assays (12). Targeted deletion of EKLF in mice results in a drastic reduction in the β-globin gene expression, but γ gene expression remains unaffected (13, 14). Finally, persistence of γ gene expression during development is observed in EKLF knockout mice (15, 16). These observations are consistent with the view that EKLF plays a central role in γ- to β-globin gene switching by binding specifically to the β promoter and providing a competitive advantage for interactions with the LCR in adult erythroid tissue. Recent studies also suggest that EKLF exerts important functions at sites other than the β-globin promoter (17, 18).

Although a large amount of information suggests a critical role for EKLF in globin gene switching, the mechanisms underlying EKLF transactivation functions are not clearly understood. EKLF is expressed, and is functional at all developmental stages, yet its effects are apparent only during definitive hematopoiesis (19–21). The molecular basis of this specificity is unknown. The dissection of EKLF functional domains is an important step in elucidating these mechanisms. Previous studies have localized a transactivation domain at the amino-terminal region (aa 1–104) (22). In this report, we describe the identification of a new transactivation domain. This domain, consisting of amino acids 140–358, is sufficient for maximal transactivation of the β-globin promoter. Furthermore, an essential region within this domain (aa 140–225) is completely redundant with the amino-terminal transactivation domain (aa 1–139).

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructions**—The HA-EKLF plasmid was constructed by subcloning a 63-base pair double-stranded oligonucleotide [top: 5′-CATGGG-TAGCAGCTACCCTTAAGACGTCGCCCGACTACGCCAGCCTGGGCGCAGGCTGGCTGGCAGTCGTAAGGGTAGCTGCTACC-3′; bottom: 5′-CATGCCCTGCTAGGGCCGCGCAGGCTGGGAGCTGGATAGCGTCTACC-3′] encompassing the HA epitope at the NcoI site of the EKLF expression plasmid pSG5/EKLF (10). This HA-EKLF plasmid was used as the
parental plasmid for the generation of deletion mutants. Restriction enzymes PvuII and Bpu10I cut in EKLF cDNA at positions corresponding to codon 3. Restriction enzymes BsaI, NarI, and AvaI cut at positions corresponding to codons 139, 225, and 254, respectively. Restriction enzyme Smal cuts at positions corresponding to codons 155 and 255. Mutant HA-Δ4-139 was generated by ligating three fragments as follows: a 0.7-kb AvrII-PvuII fragment, a 0.8-kb BsaI-BamHI fragment (BsaI end was blunted by S1 nuclease), and a 3.3-kb BamHI-AvrII fragment. Mutant HA-Δ4-225 was generated by digesting HA-EKLF plasmid with Bpu10I and NarI. The Bpu10I end was blunted with Klenow, and the NarI end was blunted with S1. The 4.6-kb Bpu10I/NarI fragment was gel-purified and self-ligated. Mutation HA-Δ4-254 was generated by digesting the HA-EKLF plasmid with Bpu10I and AvaI. The Bpu10I end was blunted with Klenow, and the AvaI end was blunted with S1. The 4.6-kb Bpu10I(AvaI) fragment was gel purified and self-ligated. Mutant HA-Δ140–226 was generated by ligation of two fragments: a 1.2-kb AvrII-BsaI fragment (the BsaI end was blunted with Klenow), and a 3.7-kb NarI-AvrII fragment (the NarI end was blunted with Klenow). Mutant HA-Δ255–358 was generated by digesting the HA-EKLF plasmid with Smal and BamHI. The BamHI end was blunted with Klenow. The 4.6-kb Smal-BamHI(blunt) fragment was gel-purified and ligated with the 300-base pair Smal fragment (codons 155–255) from EKLF cDNA. All constructs were sequenced and purified twice on cesium chloride gradients.

**Cell Culture and Transfections**—K562 cells were grown and electroporated as described previously (12). COS cells were grown in Dulbecco’s modified Eagle’s medium, 10% fetal bovine serum and transfected using coprecipitation method. Forty-eight hours after transfection, nuclear extracts were prepared, and Western blots were performed using ~50 μg of nuclear extracts that were normalized by β-gal activity. The anti-HA antibody 12CA5-HRP (Roche Molecular Biochemicals) was used at a 1:2500 dilution, and gels were developed using the ECL system (Amersham Pharmacia Biotech). Transactivation assays were performed as described previously (12). Each construct was tested in at least three independent experiments. Within each experiment, each construct was electroporated in duplicate. CAT assays were performed in duplicate on each electroporated sample, and β-gal assays were performed in triplicate on each electroporated sample. The CAT assay values were normalized by β-gal values.

**Indirect Immunofluorescence**—Indirect immunofluorescence was conducted as described previously (24), except that the cells were blocked with 10% fetal bovine serum/phosphate-buffered saline for 10 min after the permeabilization step. Coverslips were mounted in media containing propidium iodide (Vector Laboratories). 12CA5 anti-HA antibody (generous gift of Dr. Susan Ruppert) was used (1:500 dilution) as the primary antibody, and a FITC anti-mouse antibody (Santa Cruz) was used as the secondary antibody (1:500 dilution). Images were captured with a Hamamatsu CCD camera mounted on a Nikon eclipse E800 microscope.

**Gel Shift Assays**—COS cell nuclear extracts that were normalized by β-gal activity (~20 μg) as described above were used in gel shift assays using a double-stranded oligomer encompassing the mammalian β promoter CACCC box (8). The binding reactions were carried out in a buffer containing 5 mM Tris, 0.5 mM dithiothreitol, 0.5 mM EDTA, 25 mM NaCl, and 1% Ficoll. Before loading the sample on the gel, a 0.1 volume of 20% Ficoll was added to each reaction. For supershift/ablation assays, HA antibody was included in the binding reaction.

**RESULTS**

We performed a mutational analysis of EKLF using a previously established transient transfection assay (12) to map essential transactivation domains. The transactivation assay utilizes the human erythroid cell line K562, which expresses little endogenous EKLF (12), and an LCR γ/β reporter (HS2-γ/Lac-β/Cat) that contains wild-type human γ- and β-globin promoters (Fig. 1). In this assay, full-length EKLF activates β/CAT expression 500-fold compared with vector alone; therefore, the system provides a sensitive, quantitative assay for transactivation domains. We first tagged EKLF at the NH2 terminus with the influenza HA epitope (HA-EKLF) to allow detection of the protein in transfected cells. HA-EKLF transactivated the β-globin promoter in the HS2-γ/Lac-β/Cat reporter at the same level (500-fold) as wild-type EKLF. Using this parental plasmid, we constructed three progressive amino-terminal truncations of HA-EKLF: HA-Δ4-139 that deletes amino acids 4 through 139, HA-Δ4-225 that deletes amino acids 4 through 225, and HA-Δ4-254 that deletes amino acids 4 through 254 (Fig. 2A). The same methionine start site was retained for all mutants to avoid differences in the site of translation initiation. As an added control, untagged versions of each mutant were also produced and tested in the same assay; no differences between HA-tagged and untagged proteins were observed (data not shown).

Each deletion construct was cotransfected with the LCR γ/β reporter and Lac-Z control and tested for the ability to transactivate the β-globin promoter. The results are depicted in Fig. 2B. In these experiments full-length HA-EKLF (WT) activated the β-globin promoter 500-fold relative to the vector alone and is depicted as 100%. The activities of different mutants are expressed as a percentage of WT. Surprisingly, HA-Δ4-139 activated the β promoter at wild-type levels (90 ± 21%) even though this deletion removed the transactivation domain previously identified by Chen and Bieker (amino acids 1–104) (22). A further 85 amino acid deletion (HA-Δ4–225) drastically reduced β/CAT activation to 2.9 ± 2%. Deletion to amino acid 255 (HA-Δ4–254) resulted in very low levels of β/CAT activation (1.0 ± 0.6%). These data demonstrate that amino acids 140–358 encompass a potent transactivation domain that is sufficient for maximal transactivation of the β-globin promoter. Furthermore, the results suggest that an 85-amino acid subdomain (amino acids 140–225) is critical for the function of this domain.

To ensure proper integrity of the mutants, we first assessed EKLF protein levels after transfection into COS cells. Mutant constructs were cotransfected with a Lac-Z plasmid to control for transfection efficiency, and Western blots were conducted on normalized nuclear extracts using an anti-HA antibody. These results are illustrated in Fig. 3. All mutant EKLFs were detected at near wild-type levels, suggesting that the proteins were stable. We next assessed the subcellular localization of the mutants to determine whether the inactivity of HA-Δ4–225 and HA-Δ4–254 could be explained by protein mislocalization. Mutant constructs were transfected into COS cells, and HA-EKLFs were detected by indirect immunofluorescence with a FITC-labeled (green) anti-HA antibody (Fig. 4, A–E). Propidium iodide staining (red) was utilized to define the nucleus (Fig. 4, H–L), and a two-color merge (Fig. 4, O–S) was used to assess EKLF nuclear localization.
localization. As illustrated in the figure, all mutant EKLFs localized to the nucleus. These data suggest that the transcriptional inactivity of HA-Δ4–225 and HA-Δ4–254 does not result from protein mislocalization.

Finally, we examined all mutant EKLFs for the ability to bind DNA. Gel shift assays were performed with nuclear extracts obtained from COS cells following transfection of the indicated HA-EKLF mutant constructs. After 48 h, indirect immunofluorescence was performed using 12CA5 anti-HA primary antibody and FITC anti-mouse secondary antibody. Mounting medium contained propidium iodide, and cells were viewed through a fluorescence microscope using different color filters. The FITC filter depicts the nuclei expressing the HA tagged protein in green (panels A–G), Texas red filter shows propidium iodide stained nuclei in red (panels H–N), and two-color merge demonstrates colocalization (yellow, panels O–U). Representative nuclei for each construct are shown. Over 100 transfected cells for each construct were analyzed, and greater than 99% of these cells showed similar results.
Therfore, our data demonstrate that this amino-terminal domain is functionally redundant with the domain at aa 140–225. As expected, the HA-Δ140–226 EKLF protein is stable (Fig. 3), localizes to the nucleus (Fig. 4 panels F, M, and T), and binds DNA (Fig. 5, lanes 12 and 13).

**Discussion**

The results described above identify a novel transactivation domain in the erythroid-specific transcription factor EKLF. This domain, which is contained within amino acids 140–358 of the protein, is sufficient for maximal transactivation of the β-globin promoter (see HA-Δ4–139, Fig. 2B). Furthermore, an 85-amino acid subdomain of this region (aa 140–225) is essential for activity; deletion of this subdomain dramatically reduces transactivation (HA-Δ4–225, Fig. 2B). Interestingly, this subdomain can be functionally replaced by amino acids 1–139 (HA-Δ140–226, Fig. 2B), which contains a potent transactivation domain described by Chen and Bieker (22). These studies demonstrate that EKLF possesses at least two transactivation domains that function in a redundant manner. In this respect, EKLF is similar to another Kruppel-like family member, Sp1, which also contains two potent transactivation domains that are functionally redundant (25). Mutant constructs designed to delimit subregions within the 85-amino acid central domain (aa 140–225) produced unstable proteins (data not shown), precluding further definition of this domain.

Our results also demonstrate that amino acids 255–358 encompass sequences sufficient for nuclear localization; the HA-Δ4–254 EKLF mutant efficiently localized to the nucleus (Fig. 4, panels E, L, and S), although the protein did not activate transcription. To confirm this result we generated a new construct HA-Δ255–358 that deletes amino acids 255–358. This mutant was expressed at WT levels in whole cell extracts (data not shown). When tested for subcellular compartmentalization, the mutant localized exclusively to the cytoplasm (Fig. 4, panels G, N, and U). This result demonstrates that the first 254 aa of EKLF do not contain a nuclear localization sequence. The best candidate for the nuclear localization sequence is PKRGR (position 260–265). This sequence is highly homologous to the nuclear localization sequence (PKRGR) identified in other Kruppel-like family members (26).

The molecular basis for transactivation through the newly identified, central domain of EKLF is not known. However, some insight can be obtained from studies of the amino-terminal domain. The activity of this domain is regulated by phosphorylation of a conserved threonine residue within the recognition site of casein kinase II (27). EKLF is heavily phosphorylated on serine and threonine residues, and several consensus sites for phosphorylation are present within the central domain (27); therefore, the activity of this region may also be regulated by phosphorylation. Alternatively, transactivation may be a function of the proline-rich nature of this region. The activation domains of several transcription factors are rich in prolines (28), and previous studies have demonstrated that a proline stretch of 10 residues is sufficient to confer high transactivation capacity when fused to the GAL4 DNA binding domain (29). The entire EKLF protein is rich in prolines, except for the zinc finger domain (aa 275–358); therefore, both the amino-terminal and central domains may function through proline-rich sequences.

The redundancy of amino-terminal and central domains could be achieved in several ways. Both domains may interact independently with the same coactivator. This has been observed for the two redundant Sp1 activation domains, both of which interact with the same coactivator, TAFII110 (a component of the TFIID complex) (30). Alternatively, the two domains may interact with different coactivators that have re-
Novel Transactivation Domain of EKLF

dundant activities. Previous studies have demonstrated that EKLF interacts physically with CBP, P300, and P/CAF in vivo, and GATA-1 in vitro. Furthermore, Armstrong et al. (33) recently purified the SWI/SNF-related complex E-RC 1 based on its ability to interact functionally with EKLF in vitro. Additional experiments will be required to define the coactivators that interact with each EKLF transactivation domain.

As mentioned above, EKLF is phosphorylated extensively, and Zhang and Bieker (31) recently demonstrated that specific lysines are acetylated (Lys261, Lys270). These post-translational modifications may promote interactions with specific coactivators and factors of the basal transcription machinery. Differential post-translational modifications of the two transactivation domains during development may provide a mechanism for the temporal-specific transcriptional activity of EKLF on the β-globin promoter, and identification of additional EKLF interacting proteins should provide insights into globin gene switching.

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