Chromatin Structure and Transcriptional Control Elements of the Erythroid Krüppel-like Factor (EKLF) Gene*

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Erythroid Krüppel-like factor (EKLF) is a red cell-specific transcription factor whose activity is critical for the switch in expression from fetal to adult β-globin during erythroid ontogeny. We have examined its own regulation using a number of approaches. First, the EKLF transcript initiation region is in an open chromatin configuration in erythroid cells. Second, in vitro transfection assays demonstrate that the more distal of the two erythroid-specific DNase-hypersensitive sites behaves as an enhancer. Although this conserved element impacts high level transcription to a heterologous promoter in all lines examined, erythroid specificity is retained only when it is fused to the proximal EKLF promoter, which contains an important GATA site. Third, extensive mutagenesis of this enhancer element has delimited its in vitro activity to a core region of 49 base pairs. Finally, in vitro footprint and gel shift assays demonstrate that three distinct DNA binding activities in erythroid cell extracts individually interact with three short sequences within this core enhancer element. These analyses reveal that high level erythroid expression of EKLF relies on the interplay between conserved proximal and distal promoter elements that alter chromatin structure and likely provide a target for genetic control via extracellular induction pathways.

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†The abbreviations used are: EKLF, erythroid Krüppel-like factor; kb, kilobase pairs; CAT, chloramphenicol acetyltransferase; tk, thymidine kinase; bp, base pairs; oligo, oligonucleotide; LBP, Leader-binding protein; EHS, EKLF-hypersensitive site.

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conjunction with the tissue-specific proximal EKLF promoter, plays a critical role in generating high levels of expression. 

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Constructs—**32DEpo1 (22) cells were grown in Iscove’s modified Dulbecco’s medium + 10% heat-inactivated fetal calf serum and interleukin 3 (3 units/ml) or erythropoietin (2 units/ml). CV1 and NIH3T3 cells were grown in Dulbecco’s modified Eagle’s medium + 10% fetal bovine serum.

The largest EKLF construct (−3 kb) contains the promoter sequence from −2900 to +71 (BamHI to Stul murine genomic fragment (23)) in pBLCAT6 (24). The −950 construct was made by deleting the BamHI-Sacl fragment from the −3 kb construct. The −691 construct was made by deleting the XbaI-ApaI fragment from the −950 construct. The −573 construct was made by digesting the −950 construct with PsiI to remove the PsiI fragment. The −155 construct was made by digesting the PsiI-BstBI fragment from the −573 construct. The −Δ573/−155 construct was made by digesting the −950 construct to completion with BstBI, but only partially with PsiI. The second largest band was then isolated (GeneClean) and self-ligated. The −Δ686/−573 construct was made by deleting the SpaI-EcoRI fragment from the −950 construct. The −Δ686/−573 construct was made by deleting the SacII-EcoRI fragment from the −684 construct (which has the LBP-1 site). The −950 construct was made by deleting the −573 construct to completion with BstBI.

Site-directed mutants were generated by using Transformer Site-directed Mutagenesis Kit (CLONTECH) as recommended by the manufacturer. For GATA-1, the oligo primer for the opposite strand was 5′-GGTAGATGGTTGTTCTGGG-3′; oligo-4, 5′-AGCTTAGCGGTCTGAAAC3′. For LBP-1, the mutation primer was 5′-CCCTACCTG-3′.

**DNA Sequence Determination—**The DNA samples were digested with Smal and were blotted to nitrocellulose (15 μg/lane) using standard procedures (26). The 5′-end probe was the Smal-XhoI fragment, and the 3′-end probe was the SacII-Sacl fragment from EKLF cDNA (1).

**Transient Transfection and CAT Assays—**10 μg of CAT reporter and 2 μg of control growth hormone constructs were cotransfected into 1 × 10⁶ 32DEpo1 cells by the DEAE-dextran method as described (27). CV1 and NIH3T3 cells at 40–50% confluence in 100-mm dishes were transfected with 10 μg of CAT reporter and 2 μg of growth hormone control constructs by calcium precipitation (1). Calcium-DNA precipitates were added in media and incubated with cells for 7–8 h in the presence of 0.1 ml CaCl₂ per ml. After incubation, cells were centrifuged at 4 °C for another 35–40 h. CAT assays were carried out as described (27). For 32DEpo1 cells 80 μg of protein was used, and the incubation time was 2 h at 37 °C. For CV1 and NIH3T3 cells 120 μg of protein was used, and the incubation time was 1 h at 37 °C. The data from multiple experiments were averaged after normalization of CAT activity to growth hormone levels (1) and are presented as “normalized CAT activity.”

**Nuclear Protein Extraction and in Vitro Footprint Assay—**Nuclear extracts were prepared from 1 × 10⁶ 32DEpo1 cells. All buffers contained 40 μM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 10 μg/ml leupeptin, and 10 μg/ml antipain. After washing with phosphate-buffered saline + 0.35% sucrrose, cells were incubated with buffer A (10 mM Hepes, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl) on ice for 5 min. The cell pellets were then incubated in buffer A + 0.5% Triton X-100 for an additional 10 min and then transferred to TH buffer (15 mM Tris, pH 7.4, 0.35 mM sucrose, 60 mM KCl, 15 mM NaCl, 0.2 mM EDTA, 0.2 mM EGTA, 0.15 mM spermidine and 0.5 mM spermine). After spinning, the cell pellets were resuspended and incubated for 30 min on a shaker at 4 °C in buffer C (20 mM Hepes, pH 7.9, 420 mM NaCl, 1.5 mM MgCl₂, 0.7 mM EDTA, 25% glycerol). The extracted nuclei were then removed by spinning at 10,000 × g, and the supernatant was dialyzed against buffer D (20 mM Hepes, pH 7.9, 40 mM KCl, 0.2 mM EDTA, and 20% glycerol), aliquoted, frozen, and stored at −80 °C.

The template for footprint assay was generated by digesting construct HS-G with HindIII, labeling the non-encoding with Klenow fragment DNA polymerase, and performing a secondary digestion with HindIII. The DNA was isolated from a 6% polyacrylamide gel by elution, phenol/chloroform extraction, and ethanol precipitation.

**Transient Transfection and CAT Assay—**32DEpo1 nuclear protein extract in buffer containing 40 mM Hepes, pH 7.9, 110 mM KCl, 10 mM MgCl₂, 5 mM dithiothreitol, 0.05% Nonidet P-40, 17% glycerol, and 1 μg of poly(dI-poly(dC)) for each binding reaction in 50 μl. After a 1-h incubation on ice, 50 μl of Mg-Ca buffer (10 mM Hepes, pH 7.9, 1 mM MgCl₂, 0.8 mM CaCl₂) was added, and the reaction was incubated for 20 min at 30 °C. The reaction was then stopped by adding 90 μl of stop buffer (1% SDS, 20 mM EDTA, 200 mM NaCl, and 250 μg/ml yeast RNA). After phenol/ chloroform extraction and ethanol precipitation, samples were dissolved in 6 μl of loading buffer (80% formamide, 45 mM Tris base, 45 mM boric acid, 1 mM EDTA, 0.05% bromphenol blue, and 0.05% xylene cyanol) and analyzed on a 6% sequence gel.

**Oligonucleotides and Gel Shift Assay—**The oligonucleotides used for gel shift assays contained HindIII sites at each end and were as follows ("top" strand only is shown; EKLF sequence is underlined): oligo-1, 5′-GACTCAAGACGCT3′; oligo-2, 5′-GATCTATGGTTCTTCTGCGGCC3′; oligo-3, 5′-GACTCTTCTACAGG3′; oligo-4, 5′-AGCTGACGCTGAAACA′. The gel shift assay was performed as described (27). 2 × 10⁴ cpm of labeled double-stranded oligo and 0.7 μg of nuclear extract were incubated in the same buffer as used for the in vitro footprint assay, then separated on a 6% native polyacrylamide gel.

**RESULTS**

**Localization and Functional Testing of Distal EKLF Promoter Regions—**Previously it had been shown that GATA and CP-1 sites, located within 90 base pairs of the EKLF transcription initiation site, are important for activity in transient assays (21). We wished to determine whether more distal elements could augment the level of transcriptional activity seen with this minimal promoter. Our approach was to search for DNase-hypersensitive sites surrounding the EKLF transcription unit. Such open structures within chromatin can reveal sites important for enhancing transcription (28, 29). We performed these experiments using nuclei from 32DEpo1 cells (22, 30), which is a murine erythropoietic cell line that expresses EKLF,
GATA1, and β-globin. As shown in Fig. 1A, four hypersensitive sites were found, located at approximately −8.0, −0.7, −0.3, and +5.5 kb relative to the transcription initiation site. All four sites were present whether the cells were grown in interleukin 3 or erythropoietin (data not shown). To address which of these were erythroid-specific, we compared the hypersensitive status of the EKLF gene in spleens and livers from an anemic adult mouse. Earlier DNase analysis of these same samples demonstrated that only the spleen exhibits hypersensitive sites at the β-globin locus control region (25). The analyses at the EKLF promoter (Fig. 1B) demonstrate that only the two sites closest to the initiation site (at −0.7 and +0.3 kb) are erythroid-specific; the other sites (at −8.0 and +5.5 kb) are present in both tissues. The two erythroid-specific sites will be referred to as EKLF-hypersensitive sites 1 and 2 (EHS1 at −0.7 kb, and EHS2 at −0.3 kb). These sites allow us to conclude that the EKLF transcription unit resides in a more open chromatin structure in erythroid cells and also direct us to two distal locations within the promoter that may account for its tissue-specific expression.

To localize important EKLF promoter regions, we compared the upstream sequences of the murine (23) and human (31) EKLF genomic transcription units via a matrix analysis. Analysis of conserved regions has been helpful in localizing functional elements, for example the mouse and human locus control regions (32, 33). Analysis of ~800 nucleotides from the human promoter compared with ~1 kb of the murine promoter (Fig. 2) reveals that two discrete regions show extensive sequence conservation. The proximal region extends to approximately −90 and includes the GATA and CP1 sites described.
The proximal EKLF element is highly conserved in mammalian cells (Fig. 2), including the GATA and CP1 elements. We asked how alteration of these sequences would affect the ability of EHS1 to boost transcription. By using the minimal EKLF promoter construct (~77 nucleotides), we found that mutation of either site disrupted reporter levels (Fig. 5A), as previously observed (21). When EHS1 was fused to each of these constructs, all were significantly boosted (Fig. 5A). However, when normalized to their respective non-EHS1-containing values (Fig. 5B), it became clear that the GATA mutation had the most severe effect on enhancement, being boosted only ~6-fold compared with a boost of ~20-fold for the wild type and CP1 mutant. We conclude that the proximal GATA site is essential not only for minimal promoter activity but also for optimal EHS1 activity. On the other hand, the CP1 site, although important for EKLF promoter activity, is not essential for EHS1 activation.

In Vivo Analyses of EHS1—We next focused our attention on the distal EHS1 enhancer element. Inspection of the sequence surrounding the ~0.7-kb EHS1 indicated that it contains conserved GATA- (at ~684) and LBP (at ~666) (34)-binding sites. The importance of these sites was tested by directed mutationesis, and the results indicate that their disruption had no effect on the activity of EHS1 (Fig. 6A). We then tested an extensive series of deletion mutants, starting at the 5′-end with the fully active ~950 construct (Fig. 6B). Sequential deletions of approximately 20 base pairs to ~715 had no discernible effect (constructs HS-B to -J), but deletion to ~691 significantly disrupted transactivation, approaching the low level seen with the ~573 deletion. Two internal deletions then allowed us to localize the 3′-boundary of this element (Fig. 6C). Deletion of sequences from the LBP site to the ~573 boundary (~666/715) had no effect on transactivation. However, a slightly larger deletion up to ~691 (~691/753) decreased activity to the level seen with the 5′-directed ~691 deletion. These analyses suggest that full EHS1 activity is localized to the 49-bp sequence between ~715 and ~666 of the EKLF promoter.

In Vitro Analyses of EHS1—We used in vitro assays to determine which region(s) within the 49-bp sequence interact with DNA-binding proteins in extracts from 32D Epo cells. DNase footprint analysis with a 250-bp fragment from EHS1 revealed that DNase protection overlaps the 49-bp sequence of interest (Fig. 7), giving rise to regions of both decreased access
and hypersensitivity to the nuclease. These were grouped into four footprints (FP1, FP2, FP3, and FP4; Fig. 7) bound by regions of nuclease insensitivity. Note that FP1 extends beyond the 5'-boundary (−2715) of the 49-base pair sequence and that the unimportant GATA site (−684) separates FP3 and FP4.

By juxtaposing the in vitro nuclease protection and in vivo deletion data (Figs. 6 and 7), we designed four specific oligonucleotides and tested their ability to bind proteins in the 32DEpo1 extract (Fig. 8). The 5'-most sequence (oligo 1) did not bind any protein; this was not surprising as this sequence contained only half of FP1. However, the adjacent three sequences (oligos 2–4) yielded a simple pattern of interaction, each with its own single species of protein. These data led us to conclude that three DNA binding activities in erythroid cell extracts interact with the core 49-bp region of EKLF promoter DNA at approximately −700 that enhances transcriptional activation and exhibits an erythroid cell-specific open chroma-

![Figure 4](image-url)

**FIG. 4.** In vivo tests of erythroid specificity by the EKLF distal EHS1 element and the proximal promoter. A, transient transfection assays of the indicated cell lines were performed with pEHS1tkCAT or ptkCAT (pBLCAT5). Each CAT activity is expressed relative to that seen with pBLCAT5 within that particular cell line (and given an arbitrary value of 1) after normalization of data from multiple experiments. The autoradiograph of the thin layer plate from one experiment is also shown. B, transient transfection assays of the indicated cell lines were performed with the indicated expression constructs. CAT6 denotes pBLCAT6 empty vector levels and CAT5 denotes ptkCAT levels. As in A, each CAT activity is expressed relative to that seen with pBLCAT5 within that particular cell line (and given an arbitrary value of 1) after normalization of data from multiple experiments.

![Figure 5](image-url)

**FIG. 5.** In vivo tests of site-directed mutants within the proximal promoter GATA and CP1 sites in the absence or presence of EHS1. A, transient transfection assays of 32DEpo1 cells were performed with the indicated expression constructs. Solid circles denote the GATA site, and open boxes denote the CP1 site. Normalized results from multiple experiments are shown along with an autoradiograph of the thin layer plate from one experiment. B, ratios of CAT activities were obtained by dividing the normalized CAT activities shown in A as indicated.
FIG. 6. Fine-scale functional analysis of EHS1. Transient transfection assays of 32DEpo1 cells were performed with the indicated expression constructs. Normalized results from multiple experiments are shown along with an autoradiograph of the thin layer plate from one experiment in each case. A, site-directed mutants within the −684 GATA site (denoted as J) or the −666 LBP site (denoted as 2) were tested and compared with the wild type CAT activity level. B, a series of 5′-deletion mutants beginning at −950 and proceeding approximately every 20 base pairs (constructs B–J; J is deleted to −715) before ending at −691 was tested along with the full EHS1 deletion (−573). C, levels of activity of internal deletion mutants of EHS1 were compared with 5′-deletion mutants as indicated.
in vivo conformation.

In Vitro and in Vivo Tests of Putative Transcription Factor Sites within EHS1—A search for potential transcription factor sites within oligo 2 reveals that it contains overlapping glucocorticoid receptor and LBP-1 sites. However, binding to oligo 2 remains unaffected in the presence of dexamethasone or RU 486 (data not shown), rendering it less likely that the shift is due to glucocorticoid receptor. LBP (also known as LSF or CP2) binds to the long terminal repeat of human immunodeficiency virus and to the SV40 and α-globin promoters and is one of a family of related transcription factors (34–36). This protein is also unlikely to account for the oligo 2 gel shift, as anti-LSF antibodies (37) do not give rise to any supershift (data not shown). Oligo 3 contains a cytokine 2 site. NF-GMb is a cold shock domain repressor protein that binds to the cytokine 2 element in the granulocyte/macrophage colony-stimulating factor gene (38); however, it prefers binding to single-stranded DNA, an observation not seen in our studies of oligo 3 (data not shown). Oligo 4 contains sites for the UBF1 protein (an RNA polymerase I transcription factor (39)) and for a yeast α-factor responsive element (40). However, oligonucleotides that are mutated within these sites still compete as well as wild type for binding (data not shown), indicating that another protein must be responsible for the shift with oligo 4.

These data enabled us to design one final direct transactivation test of the core 49-bp EHS1. Localized mutagenesis of the three protein binding regions was performed within the context of the fully active −950 construct, and the resultant construct was tested in transfection assays. The data (Fig. 9) show that the core mutant construct is crippled for transactivation, consistent with and as expected from the deletion studies. This verifies that the three protein binding regions identified in the present study that are located between −715 and −666 are critical for optimal EKLF promoter activity.

**DISCUSSION**

EKLF is an important regulator of gene switching and β-globin expression in red blood cells. Recent studies have begun to address how the expression of such regulators are themselves controlled in erythroid cells (reviewed in Ref. 41). The present studies, summarized in Fig. 10, greatly increase our knowledge of EKLF regulation.

**The EKLF Transcription Unit Resides within an Open Chromatin Structure—**Eukaryotic transcription results from the synergistic interplay between proximal and distal promoter elements (42). These interactions are thought to result in the appropriate stereospecific structure that leads to high levels of gene activity (43). As these events occur within the confines of histones and other chromatin-associated proteins, the detection of open domains has usually correlated with actively transcribing (or “primed” (44)) areas of the genome (28, 29). Current models implicate protein-protein interactions as being of critical importance in looping such disparate regions of DNA together (46–48). In the present case, EKLF resides within a partially open structure even in cell types that are not actively transcribing it. However, nucleosome accessibility increases further only within the erythroid cell, giving rise to two specific hypersensitive sites within less than 1 kb of the start of EKLF transcription (EHS1 and EHS2; Fig. 10).

Removal of EHS2 yields a slight increase in EHS1-driven reporter activity, implicating it as a negative element that potentially targets EHS1. Regulation of an upstream activation sequence by a downstream negative regulatory site has been observed in the yeast YOR1 gene (49).

The other site (EHS1) behaves as an enhancer in transient in vivo assays and binds a limited number of proteins in erythroid
Although we have focused our attention on the erythroid-specific transcription require both this EHS1 upstream activator element and the proximal EKLF promoter, with its important GATA site. Although forced GATA1 expression could activate the EKLF proximal promoter fragment in non-erythroid cells (21), another GATA-related factor may be the actual in vivo effector of EKLF transcription, as EKLF is still expressed in the absence of GATA1 (50), and its onset of expression in development and erythropoiesis appears coincident with that of GATA1 (10, 51). In this scenario, GATA2 becomes a reasonable candidate as an EKLF effector, as it binds the same site and is suitably expressed quite early in hematopoiesis (51–53).

Although we have focused our attention on the erythroid-specific hypersensitive sites, the two constitutive sites (at −8.0 kb and +5.5 kb) may be functionally important within chromatin, possibly by providing a boundary or insulator in which the EKLF transcription unit can reside (46, 54). Studies in transgenic mice should shed light on whether these sites, in addition to the tissue-specific ones, are required together for formation of an open chromatin architecture.

The structure of the EKLF promoter is reminiscent of that of the chicken lysozyme gene (55). The chromatin surrounding this gene also contains a constitutive hypersensitive site in non-expressing cells. However, additional sites are apparent only in the chromatin within myeloblasts, which changes and become reorganized as the myeloid cells differentiate. Interestingly, formation of these hypersensitive sites is completely abolished in the absence of the lysozyme proximal promoter (55), a property also observed within the β-globin locus in erythroid cells (25) and in the rearranged immunoglobulin μ gene (56).

**Three DNA Binding Activities Interact with the Core EHS1 Element**—EHS1 gives rise to high levels of erythroid-specific transcriptional activity only in combination with the EKLF proximal promoter. The EKLF promoter contains GATA and CCAAT boxes, although it does not contain a TATA box nor an initiator element. EHS1 (bounded by −950 to −573) contains a large number of putative sites for DNA-binding proteins. Because the functional data reveal that deletions within EHS1 from either the 5'- or 3'-direction to −691 (an ApaI site) disrupt high level transcription in vivo by 50% compared with its complete deletion (to −573), sequences on either side of the ApaI site must both be required for optimal expression. At the same time, the in vitro studies indicate that there are three DNA binding activities in cell-free extracts that bind to the important 49-bp core region within EHS1 (Fig. 10). Together, these data reveal that one activity (that binds to oligo 2) binds to the 5'-side of ApaI, and two activities (that bind to oligos 3 and 4) bind to the 3'-side of ApaI. These DNA binding activities may all cooperate to synergistically activate EKLF promoter transcription, similar to that seen in other systems. For example, c-myb and CBF sites are both required for proper activity of the myeloperoxidase enhancer element (57). Similarly, Pit-1 and GATA-2 functionally cooperate within a 50-bp region to activate the thyrotropin β promoter (58). These interactions may affect the rate of EKLF transcription or the probability of forming an open chromatin structure, as in “binary” models of transcriptional control (59).

Our results have excluded a number of potential DNA binding participants in EHS1. Clearly, identifying the proteins that interact and are responsible for the enhancer properties of EHS1 will be of interest in elucidating the details of EKLF genetic regulation. Although this fragment behaved as an enhancer in all lines examined, this may not be relevant as to whether it binds ubiquitous or tissue-specific factors. For example, c-myb and CBF sites are both required for proper activity of the myeloperoxidase enhancer element (57). Similarly, Pit-1 and GATA-2 functionally cooperate within a 50-bp region to activate the thyrotropin β promoter (58). These interactions may affect the rate of EKLF transcription or the probability of forming an open chromatin structure, as in “binary” models of transcriptional control (59).

**Regulation of EKLF Expression**—The approach taken in the present studies begins at the gene locus to find the immediate cis and trans causal components of EKLF transcription. We have utilized erythropoietic cells in which EKLF is already abundantly expressed. However, it is clear that EKLF expression is induced at two very specific locations during early development (10) as follows: in the mesodermal blood islands of the yolk sac by day 7.5, and in the hepatic primordia by day 9.5. Our studies have not addressed induction mechanisms, but importantly they set the stage for future studies that will address whether the presently identified components play a role in the initial establishment of EKLF-producing cells within the early embryo, and what extracellular effectors and signal transduction pathways are involved. Of particular interest are the potential role of specific cytokine inducers that transduce via tyrosine (60, 61) or serine-threonine (62) kinase receptors.

A related issue is whether the genetic control of EKLF will differ between primitive and definitive erythroid populations. Although genetic ablation of a number of erythroid transcription factors disrupts both red cell compartments (reviewed in Ref. 63), this is not exclusively so (11–16). In addition, results of disruption of the erythropoietin receptor (17, 18) and growth responsiveness during embryonic stem cell differentiation in culture (50, 64) indicate that these two erythroid populations are regulated differently by extracellular signals. The only non-globin erythroid gene that has been analyzed at this level is that of GATA1 (reviewed in Ref. 41). In that case, transgenic studies indicate that promoter elements responsible for primiti-
The large scale view of the murine EKLF genomic locus shows its open chromatin configuration, made up of erythroid-specific EHS1 and EHS2 sites. Interactions between EHS1 and the EKLF proximal promoter give rise to high level, red cell-specific transcription. Locations of the GATA and CP1 sites within the proximal promoter are shown. The detailed view of the EHS1 region shows the colocalization in structure, sequence, and function from the present data. First, DNase hypersensitivity assays indicate that EHS1 maps to −0.7 kb relative to the EKLF transcription initiation site. Second, unlike the surrounding area, this region is highly conserved between the murine and human EKLF promoter sequences. Third, the functional footprint and electrophoretic mobility shift assay data indicate that this region can be divided into three oligonucleotide segments that each interact with a single species of DNA binding activity whose complete sequence is shown (80% identical between murine and human). Fourth, in vitro footprint and electrophoretic mobility shift assay data indicate that the core enhancer maps to the −715/−666 region, whose complete sequence is shown (80% identical between murine and human).

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