Multiple Proteins Binding to a GATA-E Box-GATA Motif Regulate the Erythroid Krüppel-like Factor (EKLF) Gene*

(Received for publication, November 14, 1997, and in revised form, February 23, 1998)

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Erythroid Krüppel-like factor (EKLF) is a zinc finger transcription factor required for β-globin gene expression and is implicated as one of the key factors necessary for the fetal to adult switch in globin gene expression. In an effort to identify factors involved in the expression of this important erythroid-specific regulatory protein, we have isolated the mouse EKLF gene and systematically analyzed the promoter region. Initially, a reporter construct with 1150 base pairs of the EKLF 5′-region was introduced into transgenic mice and shown to direct erythroid-specific expression. We continued the expression studies in erythroid cells and have identified a sequence element consisting of two GATA sites flanking an E box motif. The three sites act in concert to elevate the transcriptional activity of the EKLF promoter. Each site is essential for EKLF expression indicating that the three binding sites do not work additively, but rather function as a unit. We further show that GATA-1 binds to the two GATA sites and presents evidence for binding of another factor from erythroid cell nuclear extracts to the E box motif. These results are consistent with the formation of a quaternary complex composed of an E box dimer and two GATA-1 proteins binding at a combined GATA-E box-GATA activator element in the distal EKLF promoter.

Over the past few years, several relatively specific erythroid transcription factors have been isolated and their functional activity defined. These transcription factors regulate the expression of globin genes as well as many other erythroid-specific genes (for reviews see Refs. 1 and 2). One of these erythroid-specific factors has been termed erythroid Krüppel-like factor (EKLF)† (3). EKLF is a zinc finger DNA-binding protein that recognizes the CACCC motif in the human β-globin promoter. It has been shown that EKLF expression is restricted to the erythroid cell lineage, with initial expression in the yolk sac (4), and predominant expression occurring later in erythroid development. As a transcription factor, EKLF appears to be specifically involved in adult β-globin gene expression (4–6). Mice deficient in this gene exhibit lethal β-thalassemia (7, 8). This disease is very similar to the human β-thalassemia caused by point mutations in the CACCC sequence leading further support to the importance of the EKLF-CACCC interaction in vitro (9).

Other critical genes involved in hematopoiesis include GATA-1, Tal1, and Lmo2/ribtn2. While targeted disruption of the mouse EKLF gene results in a failure in adult erythropoiesis, inactivation of any one of these three genes, GATA-1, Tal1, and Lmo2, produces a similar phenotype characterized by a block in hematopoiesis at an earlier yolk sac stage (10–12). GATA-1 is a zinc finger transcription factor, and its expression is generally confined to erythroid cells. GATA-1 binding sites are present in all erythroid-specific genes examined to date (13). Tal1 is a basic helix-loop-helix (bHLH) transcription factor whose name is derived from its isolation at a common translocation site occurring in T-cell acute lymphoblastic leukemia (14). The protein is primarily produced in the same hematopoietic cells that also produce GATA-1. As a class B type of bHLH factor, Tal1 does not readily homodimerize but rather interacts with other HLH proteins, principally the E2A proteins, E12 and E47 (15). These heterodimers are then able to bind DNA. The general consensus site for bHLH complexes is CANNNTG and is referred to as an E box. The final erythroid-specific protein of this set, Lmo2, contains a LIM domain believed to be involved in protein-protein interactions (16, 17). Whereas this particular class of proteins also has a zinc finger motif, no evidence for direct DNA binding has been obtained.

The similar failure in erythropoiesis observed with the null mutations in each of these four genes, EKLF, GATA-1, Tal1, and Lmo2, suggest some common role or regulatory interplay in hematopoietic development. Several associations and interactions between these proteins have indeed been demonstrated. GATA-1, for example, has been shown to physically associate with both EKLF and the ubiquitous SP1 protein (18). GATA-1 is also involved in the regulation of EKLF expression through binding to a critical proximal promoter element (19). Recent developments concerning the assembly of an erythroid-specific complex of transcription factors are particularly interesting. Lmo2 and Tal1 can be found as a complex in erythroid cells (20). Subsequently it was noted that Lmo2 will also assemble with GATA-1, whereas efforts to demonstrate a stable association between GATA-1 and Tal1 were unsuccessful (21). Evidence for a model in which Lmo2 interacts with both GATA-1 and Tal1 serving as a protein link between these two DNA-binding proteins has now been provided (22). One caveat with these experiments, however, is the absence of a target gene for which these proteins, excluding GATA-1, alone or as

*This work was supported by National Institutes of Health Grant DK39555. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1794 solely to indicate this fact.

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‡The abbreviations used are: EKLF, erythroid Krüppel-like factor; Tal, T-cell acute lymphoblastic leukemia; bHLH, basic helix-loop-helix; Lmo, LIM domain only; CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility shift assay; kb, kilobase pair(s); PCR, polymerase chain reaction.
part of this large complex, have been shown to bind and effect transcriptional activity.

In our studies of the regulatory elements and associated binding factors important for temporal and tissue-specific expression of the EKLF gene, we have identified an activator element in the distal promoter region. The element consists of two GATA sites flanking an E box motif. We have shown through mutational analyses that all three sites are required for the functional activity. Such a combined site and the data suggesting the binding of multiple proteins raise interesting issues concerning the mechanisms involved in erythroid-specific gene regulation.

**EXPERIMENTAL PROCEDURES**

**Construction of Reporter Plasmids**—The basic constructs −1150EKLFcat and −124EKLFcat were prepared using PCR. Primers 5′-EKLFapk, GCTTTCCTCGACTGCTGAGTACCACC, and EKLFpcs, GAAATCCAGGCTGGGCCTGTCCTACC, were used to amplify the −1150 bp region. The EKLF3 primer and EKLF124Kp, CCTGGATCCGGACACACACATACG, were utilized to amplify the −124 bp promoter region. For transgenic animal studies, the −1150 bp KpnI-HindIII fragment was ligated 5′ of the lacZ gene containing the SV40 promoter. The HindIII fragments of −1150EKLF and −124EKLF were ligated 5′ of a CAT reporter in pGEM7Zf (Promega, Madison, WI) to yield −1150EKLFcat and −124EKLFcat, respectively. The −1150EKLFcat construct was then digested with PstI followed by religation creating Δ928−/575EKFLCAT. A KpnI-SacI fragment was eliminated to yield −952EKLFcat. −1150EKLFcat was cut in the upstream polylinker at the Spel site and at KpnI, and a 438 bp fragment was cloned in to yield −1590EKLFcat. This construct was subsequently cut with AatII and Spel to produce −1385EKLFcat.

To create mutations in the −1150 construct, a double-stranded oligomer, SpMyb, spanning −647 to −567 and containing a 5′ Apsilon overhang adjacent to an XbaI site and 3′ NcoI overhang, was ligated to the Apol and NcoI sites of −1150EKLFcat resulting in a −1150EKLFcat without the GATA-E box-GATA site. This construct was restricted with Apol and XbaI and oligomers GEG, GEMG, GEGM, and GEMGM (see Fig. 5) were inserted to make the −1150EKLFcat mutants. To produce the −60GATA mutation primers H114D, CTTATCGATCTTTTGTAAACAGCAGTAC, and the −60GATA mutagenesis oligomer, GTCCTGTCCTAGAAGCACCAGG, were used to amplify −810 to −60 mutating the GATA-E box. −60GATAEKLF were ligated 5′ of the CAT reporter plasmid and 15 μg of an SV40-luciferase control plasmid (Promega Corp., Madison, WI). Stable transfections were split into three pools and selected with G418 (Life Technologies, Inc.) at 1200 μg/ml for 4 days then maintained on 400 μg/ml. CAT assays from stables were performed with 25 μg of protein for 1 h at 37°C as described previously (27). Transient CAT assays utilized the entire transfection for 3 h at 37°C and were normalized with luciferase activity. Protein concentrations were determined using a bichinchoninic acid assay (Pierce).

**Nucleotide Sequence Accession Number**—The EKLF genomic sequence diagrammed in Fig. 1 has been deposited in GenBank®.

**RESULTS**

**The EKLF Promoter Directs Erythroid-specific Expression in Transgenic Mice**—The mouse EKLF gene was isolated from a genomic phage library using a fragment from the zinc finger region as a hybridization probe. Approximately 5.6 kb of genomic DNA was subjected to nucleotide sequence analysis; this includes 1.6 kb of 5′-flanking sequence, the 3.3-kb transcription unit, and approximately 0.7 kb of 3′-flanking region. A schematic diagram of the EKLF genomic organization is shown in Fig. 1, panel A. The gene consists of 3 exons, and a summary of the important structural features of the EKLF gene, along with the intron/exon boundary sequences, is included in panel B of Fig. 1.

For the initial characterization of the promoter activity, we sought to demonstrate erythroid-specific expression of a heterologous reporter gene driven by EKLF promoter sequences. A construct was prepared using 1150 bp of the EKLF transcription start site attached to a β-galactosidase gene. Lines of transgenic mice were established expressing this construct. Tissues, including blood, brain, lung, kidney, spleen, and testes, were collected from adult animals and assayed for β-galactosidase activity. Expression of the reporter gene was only detected in the blood samples from these mice. Bone marrow is a more active hematopoietic organ in mice (31) compared with...
**Fig. 1. Nucleotide sequence characterization of the mouse EKLF gene.** Panel A represents a scaled diagram of the mouse EKLF gene. The black boxes denote the exons; the arrow indicates the start of transcription. Specific locations of sequence features are designated in panel B. Splice sequences for each exon as compared with the consensus sequence are also included.

The 1150-bp region of the mouse EKLF promoter is thus sufficient to confer erythroid tissue-specific expression to a heterologous gene. Moreover, we mated these F1 transgenic mice to produce timed pregnancies and collected embryos at 11.5 days of gestation. The embryos were fixed and stained for β-galactosidase activity throughout the embryonic circulation and in the fetal liver. Although we did observe background staining of yolk sac tissue in the non-transgenic animals, the circulating blood is clearly indicative of erythroid-specific expression of the reporter gene.

**Identification of an Enhancer Element in the Distal EKLF Promoter**—To investigate further the regulatory elements in the EKLF promoter important for erythroid-specific expression, we chose the −124 as our basal element since Crossley et al. (19) had previously shown the presence of a critical GATA site in this proximal promoter region and indicated that this was the principal cis element within 353 bp of the transcription start site.

The fold enhancement we are reporting therefore corresponds to elevated expression compared with this 124-bp promoter with an active GATA-binding site. Comparison of the −1150 construct containing this newly described distal enhancer activity with a −77 EKLF promoter reported previously (19) in which the GATA site has been mutated results in an approximately 80-fold enhancement over this background value. We will return to a direct comparison with this proximal GATA site in a later experiment.

In addition, a non-erythroid cell line, mouse 3T3 fibroblasts, was also transfected with this series of EKLF-CAT constructs as a control to distinguish basal versus erythroid-specific expression. No expression has been observed with any EKLF-CAT construct in this non-erythroid cell line, indicating the tissue specificity evident in the transgenic mouse studies was recapitulated in our in vitro tissue culture system.

Importantly, an internal 353-bp deletion in the −1150 construct severely compromises expression from the EKLF promoter. The region missing in the Δ−928/−575 construct corresponds to a 353-bp PstI fragment. The nucleotide sequence for this region is shown in Fig. 4, panel A. We performed a computer search of this region against a database of transcription factor consensus binding sites and discovered three Sp1 sites, two GATA sites, an E box motif, a c-myb site, and an EKLF consensus site. In order to test the functional significance of these putative transcription factor binding sites, we prepared a series of CAT constructs with subfragments from this region attached to our basal −124 EKLF promoter. The designation of these clones is outlined in Fig. 4, panel B. This assay was developed as a complement to the loss-of-function analysis carried out with the −1150 internal PstI deletion. An up-regulation of activity from the EKLF −124 promoter upon inclusion of these sequences would indicate that the activator function can be added back to this basal promoter. As shown in Fig. 4, panel C, the presence of the 239-bp fragment results in a 9-fold increase in CAT activity, whereas the 114-bp fragment has no effect. This indicates that the 239-bp fragment accounts for all the activity in the element defined by deletion analysis and that this element can be moved relative to the transcription start site and still maintain its functional activity. The 239-bp fragment was subsequently further divided at the ApaI site and analyzed in a similar manner. The majority of the enhancer activity resides in the 3′-half of this fragment (Fig. 4, panel C).

**All Three Sites in the GATA-E Box-GATA Motif Define the EKLF Enhancer Element**—We noted with interest the clustering of the GATA and E box motifs and considered that alone or in combination these elements may be responsible for the erythroid-specific activation of the EKLF-CAT constructs. Double-stranded oligonucleotides flanked by restriction enzyme sites to allow easy insertion into the CAT construct were synthesized. Additionally, double-stranded oligomers were prepared in which the core E box sequence was replaced by a restriction enzyme site. Finally, an oligomer was constructed with all three sites disrupted. Mutation of either GATA site or the E box abrogated the enhancer activity of this element.
Multiple binding sites, including the E box motif and at least one of the GATA sites, are therefore necessary for the activity of this erythroid-specific enhancer element which appears to function as a unit. These experiments demonstrated that the activator could be removed from its normal position and still elevate expression from our basal EKLF promoter and that both a GATA and an E box site are important for this enhancer activity. It is possible, however, that placing this 49-bp element in the proximal promoter region allowed expression of an activity that was normally regulated by surrounding sequences, and thus our constructs did not accurately represent the impact of this element in the typical expression from the EKLF promoter. The GATA point mutations and E box replacement mutation were therefore moved into the \(-1150\) EKLF CAT construct to test the effect of these small alterations in the wild-type context. In this series of experiments, the effect of mutating each site individually and in combination was examined. The analysis of CAT activity from stably transfected MEL cells is summarized in Fig. 6. A mutation in any single site, either the 5'- or 3'-GATA sites (GMEG or GEGM) or the E box motif (GEMG), is sufficient to reduce the EKLF promoter activity to the level observed with the \(-928\) to \(-575\) deletion that originally defined this proximal enhancer. Combinations including alterations in both GATA sites (GMEGM) or all three factor binding sites (GMEGMGM) gave levels comparable to a single site mutation. All three sites thus appear to be required for the function of this distal activator.

Although the significance of these GATA-binding sites in the regulation of expression from the EKLF promoter is a novel observation, an additional GATA site in the proximal promoter region has also been shown to be important for EKLF promoter activity, as mentioned previously (19). Since the studies addressing this proximal GATA site had been carried out using constructs with only 77 bp of 5'-sequence, we wished to test the contribution of this site in the context of our \(21150\) EKLF-CAT reporter. A point mutation was therefore made in this GATA site, and the activity of this \(21150\)-(GMEG) construct was compared with the single GATA site mutations, \(21150\)-(GMEG) and \(-1150\)-(GEGM). After stable transfection in MEL cells, the normalized CAT activity from cellular extracts is shown in Table I. Clearly, all three GATA sites are critical for expression of the EKLF promoter when considered in the context of this extended 5'-promoter sequence. That is, even in the presence of an intact GATA-E box-GATA distal activator, only minimal transcriptional activity is observed if the proximal GATA site is altered. Similarly, in the framework of the 1150-bp fragment, this proximal site by itself is insufficient to direct expression of the EKLF gene.

Multiple DNA-Protein Complexes Form on the Intact GATA-E Box-GATA Motif—The previous experiments established the functional importance of the GATA-E box-GATA motif as a regulatory element in the EKLF promoter. The fact that a mutation in any single site eliminated the enhancer effect suggested that several factors could be involved in the forma-
tion of a larger protein complex, each potentially contributing to the stability through contact with its DNA-binding site. We have begun investigating the character of this putative complex with electrophoretic mobility shift assays (EMSAs) using a nuclear extract from MEL cells. With the wild-type GEG double-stranded oligomer as a probe, we regularly observe three specific complexes, labeled A, B, and C in panel A of Fig. 7.

Occasionally, slower mobility complexes are also detected, but not consistently under the particular buffer and temperature conditions used for this assay. The three major complexes can be specifically competed by the wild-type oligomer (GEG) or by any of the single mutation oligomers as shown in Fig. 7, panel A. The common factor with all these productive competitors is the presence of at least one intact GATA site.

Although a single GATA site may be all that is required to disrupt the complexes from the GEG oligomer, the formation of the A and B complexes relies on the presence of multiple sites. In panel B of Fig. 7, the binding of MEL cell nuclear factors to the wild-type GEG oligonucleotide is compared with complex formation on the probes with mutations in either the 5' - or 3'-GATA sites. The particular G → A/C mutations at these putative GATA-binding sites were prepared because previous studies (32) had shown that these nucleotide changes would prevent binding by the GATA class of transcription factors.

Although all three complexes, A–C, are observed with the GEG oligomer, only the C complex is formed when the probe carries a point mutation in one of the GATA sites. One interpretation of this result is that the C complex represents binding by a single GATA protein, and the A and B complexes contain multiple factors. The binding at each GATA site is not necessarily specific to the particular context of that GATA sequence, however. That is, binding to the GEG oligomer can be competed with the GEGM oligomer and vice versa. Nevertheless, we have noted that the GEGM oligomer appears to be less efficient in this binding reaction than either the GEG or the wild-type GEG oligonucleotide.

Whereas the results from these binding assays confirmed the importance of the GATA sites in this element, the role of the E
box was not addressed. On the one hand, a mutation in the E box motif abolished the enhancer activity, yet a specific protein-DNA complex could not be assigned to this site since the GATA sites appeared to be driving the binding activity in our assays. Therefore, as a means of focusing on the potential E box-binding protein, the GMEGM oligonucleotide was labeled and used as probe in the EMSA shown in Fig. 8. With mutations in the flanking sites precluding binding by GATA factors, a complex was formed with the central intact E box. Specificity was demonstrated by competition with the wild-type but not the mutated E box in the GATA-E box-GATA oligomers. These experiments taken together provide evidence for the formation of DNA-protein complexes at all three sites in the GATA-E box-GATA motif.

**GATA-1 Is a Component in the Complexes That Bind the EKLF Distal Enhancer**—With the important features of this cis
Antibody. In the case of the wild-type oligomer, GEG, three complexes are supershifted in the presence of the specific antibodies included in a binding assay with either the GATA-1 antibody or an antibody directed against another, unrelated protein. This experiment yielded a consensus model involving a complex of GATA-1 and a heterodimer of Tal1 and E2A bridged by Lmo2, with the newly described Ldb1 also included through an association with Lmo2. This model is based in part on previous studies demonstrating a physical interaction between GATA-1 and Lmo2 (21) and Lmo2 and Tal1 (20). If we are detecting the same type of multimeric protein structure in our studies, this would be an indication of a functional role for this complex as a transcriptional activator for erythroid-specific expression. Although the similarity in sequence configuration is compelling, there are some differences that suggest the EKLF complex may be comparable but not identical to that described by Wadman et al. (22).

One principal difference is the requirement for all three sites in our element. The 5′-GATA-binding site contributes to the functional activity of this enhancer to an equivalent degree as the 3′-GATA- or E box-binding sites. The failure to obtain a CASTing sequence with all three sites may simply be due to the fact that the oligonucleotide strands used in the procedure were composed of 26 random nucleotides, whereas the EKLF element we have described spans 49 bp.

The identity of the factor binding to the E box in the EKLF activator element is still under investigation. Based on recent evidence indicating interactions between GATA-1, Lmo2/rbtn2, and Tal1 (20–22), and the fact that Tal1 is an erythroid-specific E box factor, we have tested for the presence of Tal1 in our electrophoretic mobility shift assays. The inclusion of antibodies against either Tal1 or E2A, a known heterodimer partner for Tal1 (15), did not produce a supershift in any of the DNA-protein bands in our assays. Whereas we can detect binding at

Fig. 9. GATA-1 is a component in the complexes that bind the GATA-E box–GATA motif in the EKLF distal activator. GATA-1-specific antibodies were included in a binding assay with either the wild-type or the 5′-GATA mutant oligonucleotide probe. In each instance, the specific complexes were supershifted in the presence of the antibody. In the case of the wild-type oligomer, GEG, three complexes (A, B, and C) are affected; a single DNA-protein complex (C) is shifted in the mutant G^MEG assays. A nonspecific band (NS) is evident in all lanes and is unaffected by the antibody.

**DISCUSSION**

The EKLF Promoter Specifies Adult, Erythroid-specific Expression—A 1150-bp genomic fragment containing the EKLF promoter was shown to drive erythroid-specific and developmentally correct expression as analyzed in transgenic mice. In addition, we have transfected K562 cells with these EKLF-CAT constructs. The K562 cell line represents an earlier stage in erythroid development as compared with MEL cells. The reporter constructs are expressed in the K562 transfectants, but the levels are 40–50-fold lower than the MEL cell expression when the results are corrected for transfection efficiency between the two cell lines. This is consistent with the developmental pattern both of the reporter construct in transgenic mice and the endogenous EKLF gene. This promoter may therefore represent an avenue for producing adult, erythroid-specific expression in mice.

A GATA-E Box-GATA Enhancer Motif Resides in the Distal EKLF Promoter—This study describes the identification of an interesting configuration of binding sites in the EKLF distal promoter region that functions as a unit to elevate the transcriptional activity. The 49-bp element consists of a GATA-E box-GATA arrangement of consensus binding sites. The distal activator was functionally defined by deletion constructs in which the presence of the element consistently produced a 5–8-fold increase in reporter gene activity. Mutational analyses were carried out to demonstrate the requirement for all three binding sites, i.e. both GATA sites and the E box-binding motif. A mutation at any individual site abolishes the transcriptional activation.

Potential Assembly of a Multimeric Complex at the GATA-E Box-GATA Enhancer Element—Our results suggest the potential formation of, at the minimum, a quaternary protein complex containing two GATA-1 transcription factors and an E box dimer. These data can be considered in light of a recent paper from Wadman et al. (22) describing a large multiple protein, erythroid-specific complex in MEL nuclear extracts. These authors utilized a CASTing (cyclic amplification and selection of targets) procedure (33) to screen for preferred nucleotide sequences binding the protein of interest, Lmo2/rbtn2, in a complex with other factors. This experiment yielded a consensus site consisting of an E box and a GATA-binding site separated by 8–10 bp. This arrangement exactly matches the 3′-two-thirds of our distal activator element. The authors propose a model involving a complex of GATA-1 and a heterodimer of Tal1 and E2A bridged by Lmo2, with the newly described Ldb1 also included through an association with Lmo2. This model is based in part on previous studies demonstrating a physical interaction between GATA-1 and Lmo2 (21) and Lmo2 and Tal1 (20).

If we are detecting the same type of multimeric protein structure in our studies, this would be an indication of a functional role for this complex as a transcriptional activator for erythroid-specific expression. Although the similarity in sequence configuration is compelling, there are some differences that suggest the EKLF complex may be comparable but not identical to that described by Wadman et al. (22).

**Note:** K. P. Anderson, unpublished results.
the E box site (see Fig. 8), we have noted that the nucleotide sequence matches neither the consensus Tal1-binding site (CA-GATG) (34) nor the non-standard Tal1 site (CAGGTG) described by Wadman et al. (22). One possibility is, therefore, that an additional E box binding factor is involved. Alternatively, in this in vitro system, the binding conditions that favor binding of one protein or complex may be unsuitable for other constituents. Additional variations in buffer, temperature, and gel conditions may therefore be necessary to form and stabilize what is potentially a very large protein-DNA complex. Nonetheless, the functional activity observed in the transfected cells and the evidence for nuclear factor binding at all three sites indicate that the characterization of these binding factors will be an important avenue of continuing investigation.

Finally, an analysis of the complex formation at this distal site must consider the likelihood of protein-protein associations in addition to direct DNA binding interactions. The establishment of GATA-1 binding at this site presents intriguing possibilities in light of the expanding list of factors shown to physically associate with GATA-1. Previous studies have demonstrated interactions between GATA family members (35, 36) and between GATA-1 and EKLF or Sp1 (18). For example, a factor termed FOG was recently isolated based on its ability to bind GATA-1 (37). Although a functional role has not yet been assigned to this protein, its expression pattern mirrors that of GATA-1 and it may generally be associated with GATA in vivo.

Thus, the view of transcriptional activation is moving from simple models of factors binding to their cognate sites and individually activating transcription through an interaction with a component of the basal transcription machinery to more intricate mechanisms whereby either a protein binds to DNA and subsequently recruits additional factors into a multi-protein complex or, alternatively, the complex may assemble in the nucleus and bind en bloc to a series of binding sites. With the identification of many important transcription factors active in this system and the recent evidence for the multiple associations between these factors, studies of erythroid-specific gene regulation are expected to continue to significantly contribute to our general understanding of transcriptional mechanisms.

Acknowledgments—We thank Dr. Christine Kern for participation in the cloning of the EKLF gene during the early stages of this work and Jon Neumann for the generation of the transgenic mice. We are grateful to Dr. Richard Baer for generously providing TALI and E2A antibodies and to Dr. Emery Bresnick for kindly supplying a TAL1 antibody.

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