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The β-globin promoter is important for recruitment of erythroid Krüppel-like factor to the locus control region in erythroid cells

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ABSTRACT **Erythroid Krüppel-like factor (EKLF), which binds to the CACCC box in the β-globin promoter, is required for the expression of the β-globin gene in adult erythroid cells. It was recently demonstrated that EKLF is also required for the activity of the β-globin locus control region (LCR) 5′HS3. Some evidence suggests that the LCR and the β-globin promoter interact in adult erythroid cells, and the network of protein–protein interactions that exists between these two elements may regulate how EKLF is recruited to the LCR. In this report, we use the PIN*POINT assay to study the role of the promoter on the recruitment of EKLF to 5′HS2 and 5′HS3 of the LCR. We find that recruitment of EKLF to 5′HS2 requires the TATA box, but recruitment to 5′HS3 depends on the CACCC and TATA boxes of the β-globin promoter. Furthermore, recruitment of EKLF to 5′HS3 only occurred in β-globin-expressing murine erythroid leukemia cells, whereas recruitment of EKLF to 5′HS2 occurred in both γ-globin-expressing K562 cells and murine erythroid leukemia cells. Unlike EKLF, Sp1, which also binds to CACCC boxes, is not recruited to 5′HS3. We have also examined how one 5′HS affects the recruitment of EKLF to another 5′HS. We have found that the recruitment of EKLF to 5′HS3 depends on the presence of 5′HS2 in cis, but the recruitment to 5′HS2 does not depend on 5′HS3. Based on these results, we present a model that illustrates how EKLF may be recruited to the β-globin locus.**

Erythroid Krüppel-like factor (EKLF) is a zinc finger DNA-binding protein that is expressed in immortalized erythroid cell lines such as murine erythroid leukemia (MEL) and erythroid tissues in mouse (1–4). EKLF binds to the CACCC box in the human β-globin promoter 8-fold more efficiently than to the CACCC box in the γ-globin promoter (5) and is essential for the expression of the β-globin gene but not for the expression of the γ-globin gene. In an EKLF-null mouse embryo containing the human β-globin locus, the β-globin fails to be expressed and there is a concomitant increase in the γ-globin gene expression (6–9). In addition, the observation that a decreased level of EKLF expression in heterozygous null mice leads to higher γ-globin gene expression at the expense of β-globin gene expression supports the notion that EKLF activation of β-globin gene plays a critical role in γ–β competition and γ to β switching (9).

The role of EKLF in β-globin gene expression may occur partly by affecting the function of the β-globin locus control region (LCR), a group of erythroid-specific DNase I-hypersensitive sites (5′HS1–5) located 6–22 kb upstream of the human ε-globin gene that activates the genes in the β-globin locus (reviewed in refs. 10 and 11). In EKLF-null mice, 5′HS3 cannot function as an activator (12). By using matching point mutations (13), it was recently demonstrated that EKLF stimulated the 5′HS3 activity but that Sp1 (14) did not, despite the fact that Sp1 is also a zinc finger protein that can bind to the CACCC box *in vitro*.

Although the mechanism by which the β-globin LCR increases the expression of the β-like globin genes is still not known, early evidence from transgenic mice studies suggested that the LCR opens chromatin. The LCR conferred high position-independent expression on a linked transcription unit in transgenic mice, even when it integrated near heterochromatic regions that have suppressive effect on transcription (15). This notion was further supported by the Hispanic thalassemia, which removes approximately 35 kb, including 5′HS2–5 and 20 kb of DNA upstream of 5′HS5. This deletion results in failure to activate the β-globin locus at the levels of transcription, replication, and chromatin opening (16). More recently, however, the role of 5′HS2–5 in chromatin opening has been called into question by the observation that deletions of 5′HS2–5 significantly reduced the level of expression but did not have any effect on the general DNase I sensitivity of the native β-globin locus (17).

It is currently thought that transcriptional enhancers function by recruiting the basal transcriptional machinery to the promoter through protein–protein interaction (18, 19). Each DNase I-hypersensitive site of the β-globin LCR contains many potential binding sites for ubiquitous (e.g., Sp1 and USF) and erythroid-specific (e.g., GATA-1, NF-E2, and EKLF) transcription factors, which may be important for recruiting the basal transcriptional machinery (reviewed in ref. 10). In this context, the simplest model postulates that recruitment of the basal transcriptional machinery to the β-globin promoter occurs after the formation of the LCR complex. However, evidence from transgenic mice studies indicates that formation of the LCR complex may also be dependent on the promoter. When the promoter is rendered inactive either by mutation or by integration into suppressive chromatin region, the DNase I-hypersensitive site of the LCR is often lost (12, 20). Taken together, these findings suggest that a network of interactions, those between the DNase I-hypersensitive sites of the LCR and between the LCR and the promoter, may be important for β-globin expression. Whether such a network of interactions exists and, if so, how the network is formed, is completely unknown.

In this report, we use the PIN*POINT assay (21) to examine how EKLF is recruited to the β-globin LCR and promoter. We find that recruitment of EKLF to 5′HS2 and 5′HS3 depends on particular promoter elements, but, interestingly, the promoter elements that are required for recruitment of EKLF to 5′HS2 are different from those required for recruitment to 5′HS3. Also, the recruitment of EKLF to 5′HS3 depends on the presence of 5′HS2, but the recruitment to 5′HS2 does not depend on 5′SH3.

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Abbreviations: EKLF, erythroid Krüppel-like factor; MEL, murine erythroid leukemia; LCR, locus control region.

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EXPERIMENTAL PROCEDURES

Plasmid Construction. The expression vectors for EKLF pointers were generated by joining to EKLF cDNA to the nuclease domain of Fok I (codon optimized for expression in mammalian cells) and inserting the resulting construct downstream of both the cytomegalovirus promoter and an intron from pCIS-2 (21). Construction of the expression vector for Sp1 pointer and the target plasmid 5′HS234-β (p269) has been previously described (21). Target plasmid 5′HS234-γ was constructed by replacing the β-globin promoter of 5′HS234-β with a fragment containing the γ-globin promoter (-260 to $+36$) and a portion of the luciferase gene in pNL6 (Paul Ney, St. Jude Children's Research Hospital, Memphis, TN). The target plasmid 5′HS234-$\gamma\beta$ was generated by inserting the γ-globin promoter fragment (-260 to $+36$) between the LCR and the β-globin promoter in 5′HS234-β. Site-directed mutagenesis of the promoter was done with plasmid 5′HS234-β as a template by using the Quick-Change Site-Directed Mutagenesis Kit (Stratagene). The primers used are 5′-GCAATTTGTACTGAgctcATGGGGtCgAGAGATATATC-3′, 5′-GGTATGGGGCCAAGcttTATAcgTTAGAGGGAGG-3′, 5′-CCAACTCCTAAGtgcacGCCAGAAGAGCCAAGG-3′, 5′-GACAGGTACGGCTGTacgtACTTAGACCTCACC-3′, 5′-CATCACTTAGACCTagtaCTGTGGAGCCAC-3′, 5′-TGTGGAGCCAaAgCtTAGGGTTGGCCAATC-3′, and 5′-CCAGGGCTGGGCgagctcGTCAGGGCAGAGC-3′ for NF-1, distal GATA-1, distal CAAT, proximal GATA-1, distal CACCC, proximal CACCC, and TATA mutagenesis, respectively. The nucleotides encoding the mutation are in lowercase letters. The series of 5′HS2 mutants were made by replacing the 5′HS2 region (*Sac*I–*Bgl*II fragment) in plasmid 5′HS24-β with *Sac*I–*Bgl*II fragments from a series of plasmids containing 5′HS2 mutations that was previously published (22). The promoterless target plasmid with the LCR (p388) was constructed by cutting p269 (5′HS234-β) with *Not*I and *Eco*RI and ligating the ends after blunting them with the Klenow fragment of DNA polymerase.

Transfection and Tissue Culture. Transfection of MEL (obtained from James Bieker, Mount Sinai School of Medicine, New York) or K562 cells was done by electroporation using a Bio-Rad electroporator at 200 V and 925 μF or at 180 V and 925 μF, respectively. Tissue culture and transfected DNA recovery were performed as described previously (21).

Primer Extension. Primer extensions were performed as described previously (21). For primer extension of the β-globin promoter region, primer JS41 (5′-GGCATTTCAGTCAGTTGCTCAATGTACC-3′), derived from the chloramphenicol acetyltransferase gene, was used. For primer extensions in 5′HS2 and 5′HS3 regions, primers JS90 (5′-CCCTTCCAGCATCCTCATCTCTGAT-3′) and JS46P (5′-GGGGGTATAGGGGAGCAGTCCCATGTAGTAGTAGAATGAA-3′) were used, respectively. The annealing temperature for primers are as follows: 70°C for JS41, 72°C for primer JS46P, and 65°C for JS90. The cleavage site in the promoter, as well as in the 5′HS, was determined by running the primer extension samples next to a DNA sequencing ladder generated with the same primer.

RESULTS AND DISCUSSION

We have employed the PIN*POINT (21) assay to study and compare the recruitment of EKLF and Sp1 pointers to 5′HS2 and 5′HS3 of the β-globin LCR. In this assay, an expression vector for a fusion protein, consisting of a transcription factor of interest (EKLF or Sp1) attached to the nuclease domain of Fok I endonuclease, is transiently cotransfected with a target plasmid containing the β-globin LCR (5′HS234) and the γ- and/or β-globin promoter. Wherever the fusion protein (pointer) binds on the target plasmid, the nuclease portion cleaves the DNA, and the position and magnitude of cleavage is determined by using primer extension. Diagrams of the target plasmids and Sp1 and EKLF pointers are shown in Fig. 1*A* and *B*, respectively. Sp1 or EKLF pointer expression vector was cotransfected with one of the target plasmids into K562 (chronic myelogenous leukemia) cells, which express embryonic and fetal globin genes, or MEL cells, which express adult globin genes.

We examined the recruitment of EKLF pointer to the LCR and whether the promoter (β, γ, or both) linked to the LCR affected EKLF recruitment. As shown in Fig. 2*A*, EKLF pointer was recruited to 5′HS2 when it was linked to either β- or γ-globin promoter and in both developmental stages of the erythroid lineage (K562 vs. MEL). In contrast, recruitment to 5′HS3 was detectable for EKLF pointer (Fig. 2*B*) only in MEL cells and only in target plasmids containing the β-globin promoter (lanes 5 and 11). The summary of the EKLF pointer cleavage sites in 5′HS2 and 5′HS3 is shown below each figure.

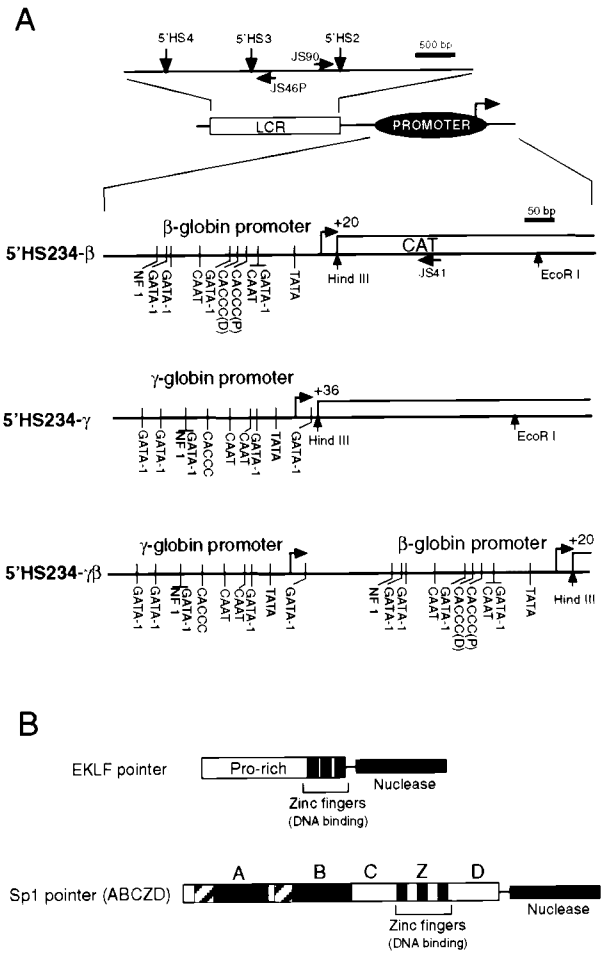

FIG. 1. (*A*) Diagram of the target plasmids. Downward arrows mark the positions of 5′HS2, 5′HS3, and 5′HS4 of the β-globin LCR (mini-LAR, mini-locus activating region), which is linked upstream of the β-globin (5′HS234-β), γ-globin, (5′HS234-γ), or tandem γ- and β-promoter. Horizontal arrows mark the positions of primers used for primer extension in this report, and vertical lines mark the positions of identified transcription factor binding sites in the β-globin promoter. Transcription initiation sites of both promoters are indicated with bent arrows. (*B*) Structure of EKLF and Sp1 pointers. The 25-kDa nuclease domain (black rectangle) of Fok I restriction endonuclease was fused to the carboxyl terminus of EKLF and Sp1. The positions of zinc fingers for EKLF and Sp1 (Z) are shown, as are the domains previously characterized for EKLF (Pro-rich, proline-rich) and Sp1 (domains A, B, C, and D).

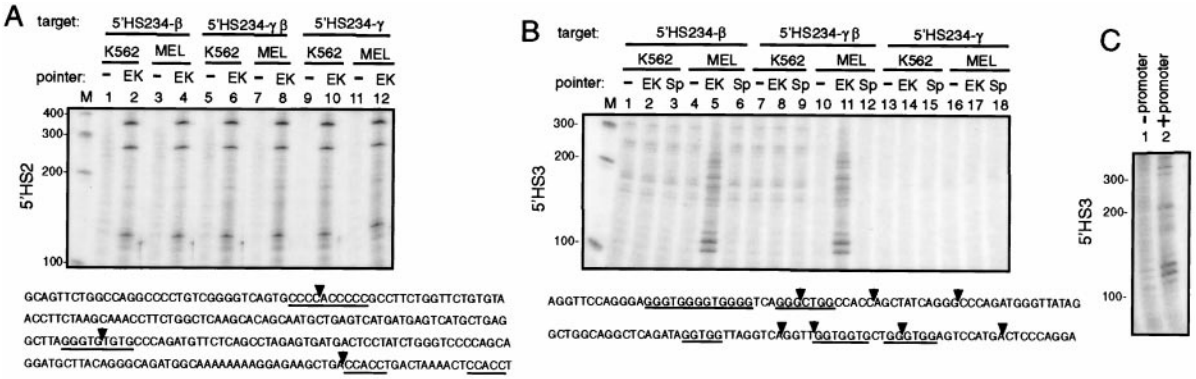

FIG. 2. The role of the β-globin promoter in EKLF pointer recruitment to the LCR. (*A*) Recruitment of EKLF pointer to 5′HS2 in the target plasmid, in which the LCR is linked to the β-globin promoter (lanes 1–4), the γ- and β-globin promoters in tandem (lanes 5–8), or the γ-globin promoter (lanes 9–12), was analyzed by PIN*POINT assay (as in Fig. 2) using the 5′HS2-specific primer JS90. The CACCC boxes (underlined) and the cleavage sites of the EKLF pointer (arrowhead) in 5′HS2 are shown at the bottom. (*B*) Recruitment of EKLF (EK) and Sp1 (Sp) pointer to 5′HS3 was analyzed by performing primer extension with 5′HS3-specific primer JS46P on the DNA samples used in *A*. The CACCC boxes (underlined) and cleavage sites (arrowheads) of the EKLF pointer in 5′HS3 are shown at the bottom. Because the bands created by the EKLF pointer were numerous and intensities slightly variable from experiment to experiment, only the cleavages that are most consistently seen are indicated at the bottom. (*C*) EKLF pointer is not recruited to 5′HS3 in the absence of a promoter in cis. The promoter in the target plasmid 5′HS234-β (+promoter, p269) was deleted and used as a target plasmid (−promoter, p388). The PIN*POINT assay was performed as in *B*.

Recently, it was demonstrated by using matching point mutations in 5′HS3 with amino acid changes in the zinc fingers of Sp1 and EKLF that in transgenic mice, EKLF activates 5′HS3, but Sp1 does not (13). However, the authors of ref. 13 were not able to determine whether the zinc finger-modified Sp1 was actually recruited to 5′HS3 but failed to activate transcription or was not recruited at all to 5′HS3. Using the PIN*POINT assay, we examined the recruitment of Sp1 to 5′HS3. We found that unlike EKLF pointer, Sp1 pointer was not recruited to the core region of 5′HS3 (Fig. 2*B*). This result suggests that one of the reasons that Sp1 does not activate 5′HS3 is that Sp1 may not be recruited to 5′HS3.

From these findings, it is not possible to determine whether the recruitment of EKLF to 5′HS2 does not depend on a promoter in cis or whether the promoter is required in cis but either γ- or β-globin promoter is sufficient. Also, we cannot rule out the possibility that the reason that EKLF is not recruited to 5′HS3 when it is linked to the γ-globin promoter is that the γ-globin promoter suppresses EKLF recruitment to 5′HS3. To test these possibilities, we examined the recruitment of EKLF to 5′HS2 and 5′HS3 in a target plasmid with the LCR (5′HS2, -3, and -4) but no promoter. EKLF pointer cleavage in 5′HS2 (data not shown) and 5′HS3 (Fig. 2*C*) was significantly decreased in the promoterless target plasmid. We conclude from this result that the recruitment of EKLF to 5′HS2 is enhanced by the presence of a promoter (γ- or β-globin), and the recruitment of EKLF to 5′HS3 is enhanced by the presence of the β-globin promoter in cis. This is consistent with an LCR–promoter interaction and implies that transcriptional complex formed on the respective promoters stabilizes or supports recruitment of EKLF to the LCR.

These observations prompted us to examine more closely the role of the individual transcription factors binding to the β-globin promoter. This was done by mutating each transcription factor binding site in target plasmid 5′HS234-β individually (described in Fig. 3*E*) and analyzing the recruitment of EKLF pointer to the promoter, 5′HS2, and 5′HS3 in the promoter-mutated target plasmids. The mutation of the proximal CACCC box and the TATA box abolished the recruitment of EKLF pointer to the promoter (Fig. 3*A*, lanes 6 and 7) and 5′HS3 (Fig. 3*C*, lanes 6 and 7), but only the mutation of the TATA box abolished the recruitment to 5′HS2 (Fig. 3*B*, lane 7). That recruitment of the EKLF pointer to 5′HS2 and 5′HS3 is supported by the promoter may help explain why the formation of DNase I-hypersensitive sites in the LCR may depend on the promoter in cis (12, 20).

To compare the transcriptional activity of the mutant promoters with the status of EKLF recruitment to the LCR and the respective promoter, we measured the chloramphenicol acetyltransferase activity after transiently transfecting the target plasmids into MEL cells. As shown in Fig. 3*D*, the β-globin promoter activity was significantly reduced by the mutation of the proximal CACCC box but was still detectable. The β-globin promoter activity was abolished by the mutation of the TATA box. Thus, recruitment of EKLF to the β-globin promoter correlates with the β-globin promoter activity.

Although recruitment of EKLF to both 5′HS2 and 5′HS3 is supported by the promoter in cis, these results suggest that the level of support from the promoter that is required for EKLF recruitment to 5′HS2 may be different from the level of support required for EKLF recruitment to 5′HS3. Because recruitment of EKLF to 5′HS3 occurs only in MEL cells and requires the promoter elements necessary for transcription of the β-globin gene, recruitment of EKLF to 5′HS3 may require the support of a stable preinitiation complex formed on an active promoter. On the other hand, recruitment of EKLF to 5′HS2 occurred even when the proximal CACCC box in the β-globin promoter was mutated. Without the proximal CACCC box, the activity of the β-globin promoter was significantly reduced but still detectable. Without the TATA box, there was no recruitment of EKLF to 5′HS2 and no detectable activity of the β-globin promoter. This suggests that the preinitiation complex formed on the CACCC boxless β-globin promoter, albeit very unstable, may be sufficient to support recruitment of EKLF to 5′HS2. One possible implication of this finding is that recruitment of EKLF to 5′HS2 may be intrinsically more stable than recruitment of EKLF to 5′HS3.

The activity of the 5′HSs is additive, and full expression of the β-globin gene cluster requires all 5′HSs (23–28). Although the individual 5′HSs may act independently of one another, it has also been proposed that the complexes binding to each 5′HS synergize by interacting to form an LCR holocomplex (27, 29–31). If such a holocomplex is formed at the LCR, recruitment of proteins such as EKLF to one 5′HS is likely to depend on the other 5′HS. We tested whether such dependence exists by using target plasmids containing either 5′HS2 or 5′HS3 alone or 5′HS2 and 5′HS3 or 5′HS2, -3, and -4 together. As shown in Fig. 4, recruitment of EKLF pointer to 5′HS2 does not depend on the presence of 5′HS3 (Fig. 4*A*), but

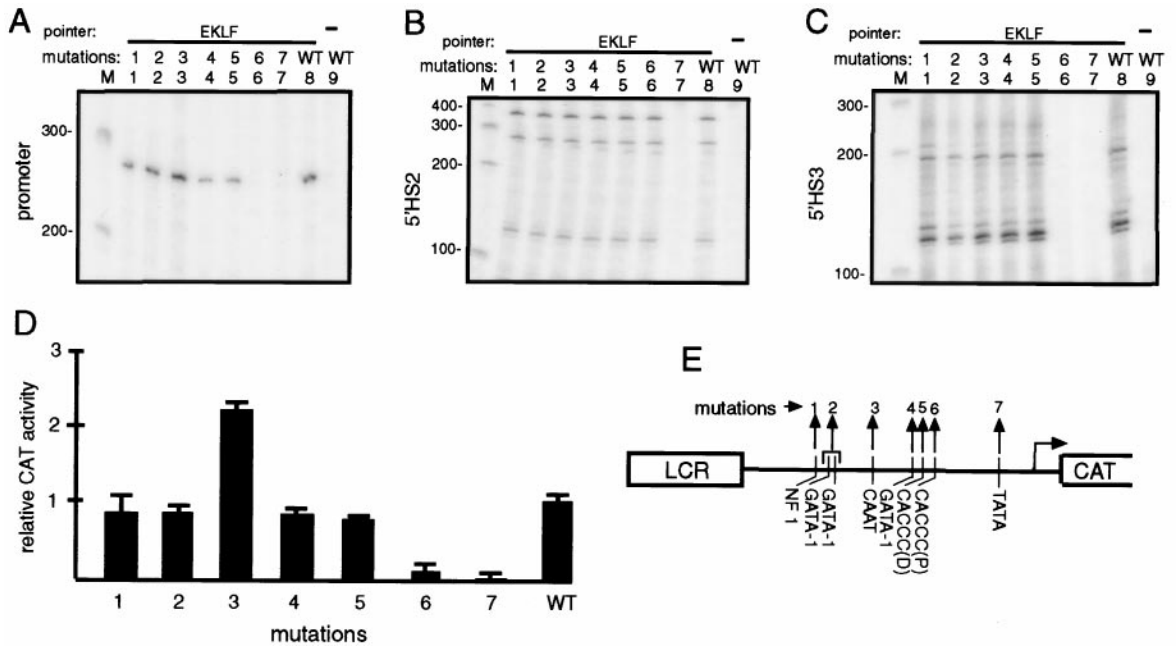

FIG. 3. The role of various promoter elements in EKLF pointer recruitment. A series of promoter-mutated target plasmids derived from 5′HS234-β (described in *E*) were cotransfected with EKLF pointer expression vector. Recruitment of EKLF pointer to the β-globin promoter (*A*), to 5′HS2 (*B*), and to 5′HS3 (*C*) of each of these target plasmids was analyzed by primer extension using primers JS41 (*A*), JS90 (*B*), and JS46P (*C*). Lanes 1–7 correspond to target plasmids with mutation 1–7, lane 8 to wild-type promoter, and lane 9 to no pointer expression vector. (*D*) The transcriptional activity of the β-globin promoter constructs containing the mutations shown in *E*. The target (reporter) plasmids (10 μg) were transiently transfected into MEL cells, and chloramphenicol acetyltransferase (CAT) assay was performed after 48 h. (*E*) The mutated transcription factor binding sites in the β-globin promoter of the target plasmids used in panels *A–C* are shown schematically.

recruitment to 5′HS3 depends on 5′HS2 (Fig. 4*B*), as does recruitment of EKLF to the promoter (Fig. 4*C*). This is consistent with our earlier finding (Fig. 3), which suggested that recruitment of EKLF to 5′HS2 may be more stable than recruitment of EKLF to 5′HS3.

Because EKLF is recruited to 5′HS2 independently of 5′HS3 or the CACCC box in the β-globin promoter, it is possible that 5′HS2 is more promiscuous than 5′HS3 or the β-globin promoter. As such, EKLF may be recruited to 5′HS2 through protein–protein interaction. This is unlikely to be the case because each of the three cleavage sites in 5′HS2 is lost if the corresponding CACCC box is deleted (data not shown).

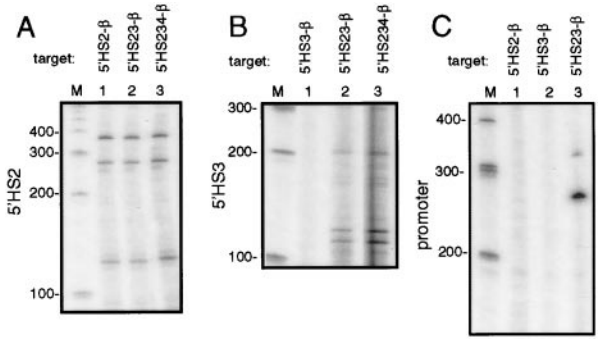

FIG. 4. Recruitment of EKLF pointer to 5′HS3 requires 5′HS2. (*A*) A target plasmid containing 5′HS2 (lane 1), 5′HS23 (lane 2), or 5′HS234 (lane 3) linked to β-globin promoter was cotransfected with the expression vector for EKLF pointer. Primer extension was performed with 5′HS2-specific primer JS90. (*B*) A target plasmid containing 5′HS3 (lane 1), 5′HS23 (lane 2), or 5′HS234 (lane 3) linked to the β-globin promoter was cotransfected with the expression vector for EKLF pointer. Primer extension was performed with 5′HS3-specific primer JS46P. (*C*) The DNA samples used in lane 1 (5′HS2-β) of *A* and lanes 1 (5′HS3-β) and 2 (5′HS23-β) of *B* were subjected to primer extension with a promoter-specific primer JS41 for lanes 1, 2, and 3, respectively.

It is more likely that the complex formed on 5′HS2 has a strong transcription factor recruitment activity that 5′HS3 does not have. Indeed, 5′HS2, but not 5′HS3, can act as an enhancer in transiently transfected cells (32–36). Furthermore, deleting 5′HS2 in yeast artificial chromosome constructs containing the entire β-globin locus severely decreased the hypersensitive site formation and β-globin gene expression in transgenic mice (31). Replacing 5′HS2 with 5′HS3 restored the hypersensitive site formation but not β-globin gene expression, suggesting that 5′HS2 has a unique enhancer activity.

Together with other results (C.-H.L., M. R. Murphy, J.-S.L., and J.H.C., unpublished work), the simplest interpretation of the findings presented here is that the 5′HSs of the LCR and the promoter together form a holocomplex. This holocomplex may also include a switch/sucrose nonfermenting (SWI/SNF)-like chromatin-remodeling complex such as EKLF coactivator-remodeling complex 1 (ref. 37, and Fig. 5), and histone acetyltransferases such as p300 activate the β-globin promoter through an interaction with EKLF (38). Because p300 acetylates not only transcription factors such as EKLF but also histones, it appears that diverse chromatin-remodeling activities may participate in β-globin expression.

The observation that cleavage by EKLF pointer was not detectable if the TATA box was mutated is intriguing. Because the probability of cleavage by the nuclease tail depends on the length of time the EKLF pointer stays bound to DNA, it is likely that, in general, the PIN*POINT assay detects primarily stably recruited EKLF pointer. This would imply that *in vivo*, the TATA-binding protein (reviewed in ref. 39) and/or the other components of the preinitiation complex help stabilize EKLF on the β-globin promoter and the LCR through protein–protein interaction.

Transcription is a very complex event involving a network of protein–protein interactions that recruits the RNA polymerase II (40). Despite the vast knowledge accumulated over the past two decades in this area, how individual transcription factors are recruited *in vivo* to their binding sites is virtually unknown.

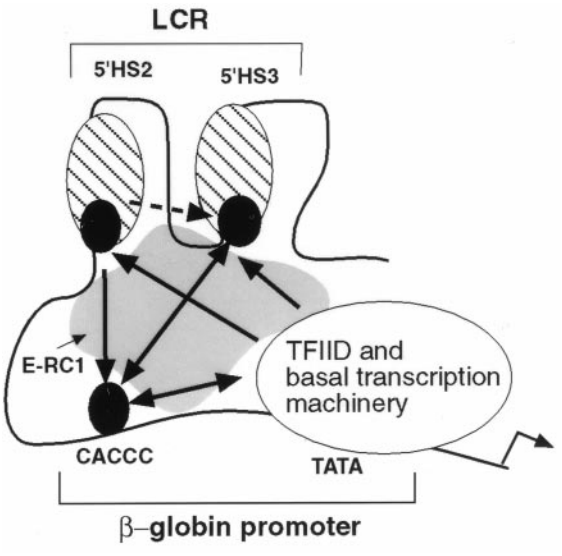

FIG. 5. The recruitment of EKLF depends on a network of interactions between the LCR and the β-globin promoter. Arrows indicate that a protein or a protein complex was required for the recruitment of EKLF (black oval) to either 5′HS2 and 5′HS3 or the β-globin promoter. The arrows point to the recruited protein. Double-headed arrows indicate mutual dependence. The dashed arrow indicates that the recruitment of EKLF to 5′HS3 depends on the presence 5′HS2. The arrows only indicate dependence for recruitment as revealed by the PIN*POINT assay performed in this and other reports (C.-H.L., M. R. Murphy, J.-S.L., and J.H.C., unpublished work) and do not show all potential dependence for recruitment or imply direct interaction. The role of EKLF on recruitment of TATA-binding protein (TFIID) was derived from the results in another report (C.-H.L., M. R. Murphy, J.-S.L., and J.H.C., unpublished work). The striped oval indicates the complex on the 5′HSs, and the gray object indicates the SWI/SNF-related complex, such as EKLF coactivator-remodeling complex (E-RC1). The transcription initiation site is indicated with a bent arrow.

In this and other reports (C.-H.L., M. R. Murphy, J.-S.L., and J.H.C., unpublished work), we have begun to elucidate the network of protein–protein interactions controlling the recruitment of EKLF to the β-globin LCR and promoter and the interdependence between the LCR and the promoter in this process. Although the PIN*POINT assay may not reflect all aspects of *in vivo* protein recruitment precisely, what we have presented in this report serves as a framework toward a better understanding of how transcription complexes are formed *in vivo*. Additional work, such as studying the transcription factor recruitment to the endogenous β-globin locus, will be needed before we can understand how transcription actually occurs *in vivo*.

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