Erythroid Krüppel-like factor is recruited to the CACCC box in the β-globin promoter but not to the CACCC box in the γ-globin promoter: The role of the neighboring promoter elements

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Edited by Gary Felsenfeld, National Institutes of Health, Bethesda, MD, and approved December 23, 1999 (received for review November 3, 1999)

The programmed expression of the five β-like globin genes (ε, ^{A}γ, ^{G}γ, δ, and β) is characterized by a series of switches that are developmentally regulated. The ^{A}γ- and ^{G}γ- (fetus) to β-globin (adult) switch depends on transcription factor erythroid Krüppel-like factor (EKLF), which, like Sp1, binds to CACCC boxes. EKLF is essential for the expression of the β-globin but not the γ-globin gene. Because both γ-globin and β-globin promoters contain the CACCC box, and their promoter elements are similar, it is not known why the two promoters behave so differently. In this report, we searched for the functional differences between the two promoters by studying their ability to recruit EKLF. We used the *in vivo* PIN*POINT assay to show that EKLF is recruited to the β-globin promoter but not to the γ-globin promoter. We show that this selectivity is a result of differences in surrounding promoter elements and not CACCC box alone. One of the differences between the two promoters with a functional consequence is the CCTTG repeat that is present in the γ-globin promoter but not in the β-globin promoter. The repeat, when inserted in the β-globin promoter, decreases EKLF recruitment to and activity of the β-globin promoter, suggesting that the repeat functions as a suppressor element. The CCTTG repeat can also suppress the SV40 promoter in cis, and the suppressor factor binding to the repeat can be squelched with a plasmid containing a high copy number of the repeat. These findings may have implications in designing drug targets for treatment of β-globin disorders.

Globin gene expression is limited to the cells of the erythroid lineage and undergoes developmentally programmed switching (reviewed in refs. 1–5). There are five β-like globin genes (ε, ^{A}γ, ^{G}γ, δ, and β) in humans, and their expression switches from ε (embryo) to ^{A}γ and ^{G}γ (fetus) and finally to δ and β (adult). The expression of the five β-like globin genes is regulated not only by their promoter elements but by DNase I hypersensitive sites called locus control region (LCR) located near the 5′ end of the β-globin domain.

The promoter regions of the β-like globin genes contain many common cis-acting elements. One of them is the CACCC box, which has been shown to be important for the expression of the γ- and β-globin promoters. Naturally occurring mutations of the CACCC box in the β-globin promoter cause β^{+} thalassemias (6), and deletion of the CACCC box in the γ-globin promoter significantly reduces activity of that promoter (7, 8).

Sp1-related transcription factors including Sp1(9) and erythroid Krüppel-like factor (EKLF) (10) can bind to the CACCC box. The expression of EKLF is restricted to the cells of the erythroid lineage, but the expression of Sp1 is ubiquitous. Even though EKLF is present in erythroid cells of all developmental stages and the CACCC box is present in the promoters of embryonic ε-, fetal ^{A,G}γ-, and adult β-globin genes, only β-globin gene expression is severely reduced in EKLF null mice (11, 12). With the decrease in β-globin, there was a concomitant increase in the γ-globin gene expression in transgenic mice with integrated human β-globin locus (12, 13).

Both γ- and β-globin promoters contain CAAT and CACCC boxes and GATA-1 and NF1 binding sites in roughly similar locations of the promoter. Despite the similarity between the two promoters, EKLF is essential for β-globin gene expression but not for γ-globin gene expression. This may be because EKLF has eight-fold greater affinity for the proximal CACCC box in the β-globin promoter (CCACACCC) than for the CACCC box in the γ-globin promoter (CTCCACCC) (14). However, replacing the CACCC box in the γ-globin promoter with that from the β-globin promoter or replacing the CACCC boxes in the β-globin promoter with that from the γ-globin promoter made no difference in whether the promoter is activated or not activated by EKLF (15). The promoter context, rather than the CACCC box itself, seems to be the determinant of EKLF responsiveness. Based on these observations, it was proposed that the basal transcriptional machinery recruited by EKLF activates the β-globin promoter but not the γ-globin promoter (15). However, the effect of EKLF on the two promoters has always been measured by its ability to activate transcription. Therefore, it has not been possible to distinguish whether the failure of EKLF to activate the γ-globin promoter was a result of EKLF not being recruited to the γ-globin promoter or a result of the basal transcriptional machinery recruited by EKLF not being able to activate the γ-globin promoter.

In this report, we use the PIN*POINT assay (16–19) to study the recruitment of EKLF to γ- and β-globin promoters. We find that the reason EKLF does not affect the γ-globin gene expression is that it is not recruited to the γ-globin promoter. In addition, we have identified a 31-bp region in the γ-globin promoter containing four repeating copies of CCTTG that suppresses EKLF recruitment. The repeat appears to mediate its suppressor activity through a trans-acting factor that can be squelched with a plasmid containing a high copy number of the repeat.

Methods

Plasmid Construction. The expression vectors for Sp1 and EKLF pointers were generated by joining Sp1 and EKLF cDNAs to the

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: LCR, locus control region; EMSA, electrophoretic mobility-shift assay; EKLF, erythroid Krüppel-like factor.

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Article published online before print: *Proc. Natl. Acad. Sci. USA*, 10.1073/pnas.040476297. Article and publication date are at www.pnas.org/cgi/doi/10.1073/pnas.040476297

nuclease domain of FokI (codon optimized for expression in mammalian cells) and inserting it downstream of the CMV promoter and an intron from pCIS-2 (19). Target plasmids 5′HS234-β (16) and 5′HS234-γ (19) have been described previously. Target plasmid 5′HS234-$\gamma\beta$ was constructed by inserting the γ-globin promoter fragment (-260 to $+36$) into the *Not*I site between the LCR and the β-globin promoter. Chimeric promoter 1 was constructed by swapping the γ-globin CACCC box with the β-globin CACCC box by using the Quick-Change Site-Directed Mutagenesis Kit (Stratagene). The primers used are 5′-GTCCCTGGCTAAACcaCACCCtTGGGTTGGCCA-GC-3′ and 5′-GCTGGCCAACCCAaGGGTGtgGTTTAG-CCAGGGAC-3′. Altered nucleotides are in lower case letters. Chimeric promoters 2 and 3 were constructed by introducing a *Bgl*II site immediately upstream of the γ- and β-globin TATA boxes and exchanging the minimal promoter regions of the two promoters. To construct chimeric β-globin promoters 4–7, a *Bgl*II site was first introduced between the proximal CACCC box and the proximal CAAT box of the wild-type β-globin promoter in 5′HS234-β. Double-stranded oligonucleotides shown in Fig. 3*C*, which contained a 5′ GATC overhang on both strands (overhangs not shown) were inserted into the *Bgl*II site.

The reporter construct $\gamma\Delta$-luc (p793) was constructed from pNL6 by using the Quick-Change Site-Directed Mutagenesis Kit (Stratagene) and oligonucleotides JS114d (5′-CTATTGGT-CAAGTTTTGGCCAACCCATGGG-3′) and JS115d (5′-CCCATGGGTTGGCCAAAACTTGACCAATAG-3′). The reporter construct γ-luc is the same as pNL6. Competitor plasmid $(CCTTG)_{15}$ was constructed by annealing oligonucleotides JC434 (5′-AGCTTGCCTTGCCTTGCCTTGCCTTGC-CTTGCCTTGCCTTGCCTTGCCTTGCCTTGCCTTGC-CTTGCCTTGCCTTGCCTTGA-3′) and JC435 (5′-AGCT-TCAAGGCAAGGCAAGGCAAGGCAAGGCAAGGCA-AGGCAAGGCAAGGCAAGGCAAGGCAAGGCAAGG-CAAGGCAAGGCA-3′) and inserting the annealed oligonucleotides into the *Hin*dIII site of pBlueScript SK(+) (Stratagene). Competitor plasmid $(CCGAG)_{15}$ was constructed in the same way by using oligonucleotides DK3 and DK4, which contain CCGAG repeats. Reporter construct SV40(4R)-luc was constructed by inserting the double-stranded oligonucleotide WTx4 (HN 47: 5′-GATCGAATTCGCCTTGCCTTGCCTTG CCTTG-3′ and HN 48: 5′-GATCCAAGGCAAGGCAAG-GCAAGGCGAATTC-3′) into the *Bgl*II site 5′ of the SV40 promoter in pGL3 (Promega). Reporter construct SV40(2R)-luc was constructed by inserting annealed oligonucleotides DK5 (5′-CGCGTAATTCGCCTTGCCTTGGATGCGGACC-3′), and DK6 (5′-TCGAGGTCCGCATCCAAGGCAAGGC-GAATTA-3′) into Mlu I and *Xho*I sites of pGL3. Reporter construct SV40-luc is same as pGL3. The expression vector for COUP TF II was constructed by inserting the mouse COUP TF II cDNA (M. J. Tsai, Baylor College of Medicine, Houston, TX) into the *Eco*RI and *Xho*I sites of pCDNA3 (Invitrogen).

Transfection. For PIN*POINT and chloramphenicol acetyltransferase assays, transfection was performed as described in ref. 16. For transiently transfecting luciferase-expressing reporter constructs, Effectene Transfection Reagent (Qiagen, Chatsworth, CA) was used following the manufacturer's recommendations. For transfections using the luciferase reporter constructs, 0.02 μg of the reporter construct and 0.18 μg of the competitor plasmid [pBlueScript SK(+), $(CCTTG)_{15}$ or $(CCGAG)_{15}$] for K562 cells ($0.5–1.0 \times 10^6$). For transfecting MEL cells ($0.8–1.2 \times 10^6$), 0.04 μg of the reporter construct and 0.16 μg of competitor plasmid were used. Luciferase assays were performed with the Luciferase Assay System (Promega) 48 hr after transfection. To express COUP TF II in COS-7 cells, 0.1 μg of the COUP TF II expression vector or empty pCDNA3 vector (Invitrogen) was used.

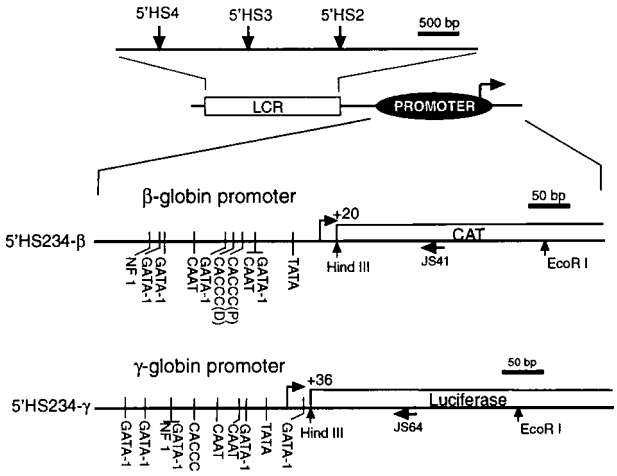

Fig. 1. A diagram of the target plasmids. Downward arrows mark the positions of 5′HS2, -3, and -4 of the β-globin LCR (mini-LAR), which is linked upstream of the β- globin (5′HS234-β) and γ-globin (5′HS234-γ) promoters. Horizontal arrows mark the positions of primers used for primer extension in this report, and vertical lines mark the positions of identified transcription factor binding sites in the β- and the γ-globin promoters. Transcription initiation sites of both promoters are indicated with bent arrows.

Electrophoretic Mobility-Shift Assay (EMSA). Binding reactions were performed with 1–2 μg of MEL and K562 nuclear extract in a binding buffer (5×) composed of 20% glycerol, 5 mM $MgCl_2$, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, 50 mM Tris·HCl (pH 7.5), and 0.25 mg/ml poly(dI-dC) (Pharmacia). Probes (0.1 pmol) were 5′ end-labeled with T4 polynucleotide kinase by using [γ-^{32}P]ATP. For binding reactions using COUP TF II expressing cell extract, 2–4 μg was used. The binding reaction and electrophoresis were performed at room temperature.

Primer Extension. Primer extensions were performed as described (16). For primer extension of the β- globin promoter region, oligonucleotide JS41 (5′-GGCATTTCAGTCAGTTGCT-CAATGTACC-3′) derived from the chloramphenicol acetyltransferase gene was used. For primer extension through the γ-globin promoter region, oligonucleotide JS64 (5′-CTTTCTT-TATGTTTTTGGCGTCTTCCATTTTACC-3′) from the luciferase gene was used. The primers were annealed at 70°C before primer extension.

Results

We employed the PIN*POINT (16) assay to compare the recruitment of EKLF and Sp1 pointers to γ- and β-globin promoters. To do this, Sp1 or EKLF pointer expression vector were cotransfected with one of the target plasmids into K562 (chronic myelogenous leukemia) or MEL (murine erythroleukemia) cells, and the target DNA was isolated after 24–36 hr. The cleavage in the target DNA was visualized by primer extension with primer JS41 for the β-globin promoter and JS64 for the γ-globin promoter (Fig. 1). Although transcription factor binding sites in the two promoters are grossly similar, EKLF pointer was recruited only to the β-globin promoter (Fig. 2*A*, compare lanes 2 with 8 and 5 with 11). Sp1 pointer, in contrast, was recruited to both promoters in K562 and MEL cells (Fig. 2*A*, lanes 3, 6, 9, and 12). The cleavage site was determined to be 8 bp upstream of the distal CACCC box in the β-globin promoter for both pointers (Fig. 2*A*, bottom). According to this result, the reason EKLF does not activate the γ-globin promoter is not that it fails to recruit an active basal transcriptional machinery on the γ-globin promoter but rather that it is not efficiently recruited to

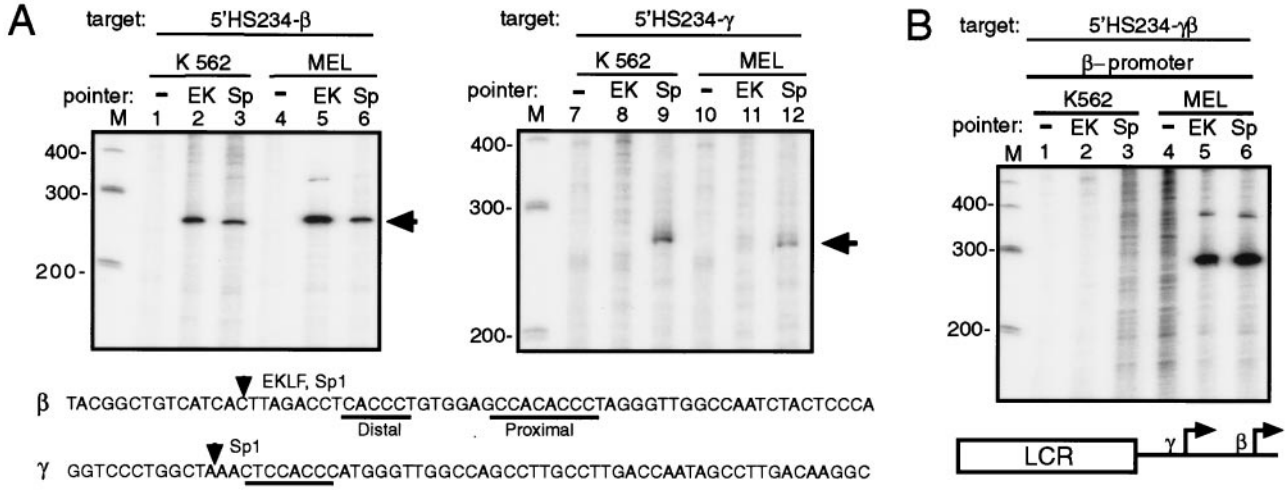

Fig. 2. An analysis of EKLF recruitment to the γ- and β-globin promoters. (*A*) EKLF is recruited to the β-globin promoter but not to the γ-globin promoter. EKLF (EK), Sp1 (Sp), or no (-) pointer expression vector was cotransfected with target plasmids 5′HS234-β (lanes 1–6) or 5′HS234-γ (lanes 7–12) into either K562 or MEL cells as indicated. Twenty-four to thirty-six hours after transfection, the target plasmid was isolated, and the cleavage site in the target plasmid was determined by performing primer extension with primers JS41 (lanes 1–6) or JS64 (lanes 7–12). The cleavage-induced band is marked with a horizontal arrowhead. The cleavage sites (vertical arrowhead) in the β-globin promoter for both pointers and in the γ-globin promoter for Sp1 pointer are shown below. The CACCC boxes for both promoters are underlined. (*B*) Recruitment of EKLF in the context of γ-β competition. EKLF, Sp1, or no (-) pointer expression vector was cotransfected into either MEL or K562 cells with target plasmid 5′HS234-γβ (shown below) in which the γ-globin promoter region (−260 to +36) was inserted between the LCR and the β-globin promoter. Recruitment of EKLF or Sp1 pointer to the β-globin promoter was detected by performing a primer extension with primer JS41.

the γ-globin promoter. Sp1, on the other hand, is recruited to the β-globin promoter. However, the observation that Sp1 does not significantly activate the β-globin promoter in a similar type of transient cotransfection experiment (20) suggests that Sp1 either fails to recruit the basal transcriptional machinery or that the basal transcriptional machinery recruited is inactive for β-globin promoter. Therefore, the reason why EKLF does not affect γ-globin expression appears to be different from why Sp1 does not affect β-globin.

In their genomic context, γ- and β-globin promoters have been proposed to compete for the LCR (1–5, 21). In fetal erythroid cells, the γ-globin gene is preferentially expressed, and, in adult erythroid cells, the β-globin gene is preferentially expressed. To study EKLF and Sp1 recruitment in the context of γ-β competition, both promoters were linked to the LCR on the same plasmid (5′HS234-γ-β) and were used as a target plasmid in a PIN*POINT assay. In K562 cells, which behave like early fetal erythroid cells and express the γ-globin gene, neither the Sp1 pointer nor the EKLF pointer was recruited to the β-globin promoter (Fig. 2*B*, lanes 1–3). As expected from the result above, the Sp1 pointer but not the EKLF pointer was recruited to the γ-globin promoter in K562 cells (data not shown). In MEL cells, which behave like adult erythroid cells and express the β-globin gene, both EKLF and Sp1 pointers were recruited to the β-globin (Fig. 2*B*, lanes 4–6) but not to the γ-globin promoter (data not shown). These findings suggest that the recruitment of EKLF is not only restricted to the β-globin promoter but is sensitive to the developmental stage of the erythroid cell. *In situ* hybridization experiments have shown that the LCR activates only one gene at a time; γ-globin gene is selected in fetal erythroid cells, and the β-globin gene is selected in adult erythroid cells (22). Taken together with the *in situ* hybridization experiments, our findings indicate that EKLF is recruited most efficiently in the cellular environment where the LCR- β-globin interaction is taking place.

To determine why EKLF pointer is not recruited to γ-globin promoter, we searched for functional differences between the γ-globin and β-globin promoters. One of the differences is in the sequence of the CACCC box: CTCCACCC in the γ-globin promoter and CCACACCC in the β-globin promoter (14). The β-globin promoter CACCC box has an eight-fold greater affinity for EKLF than the γ-globin promoter CACCC box. Another difference is the presence of a 31-bp region containing four repeats of CCTTG immediately downstream of the CACCC box in the γ-globin promoter that is not present in the β-globin promoter (Fig. 3*A*, striped rectangle). Two copies of CCTTG repeats are also present downstream of the CACCC box in the rabbit γ-globin promoter (Fig. 3*B*). The role of these repeats in γ-globin expression has not been defined.

We first examined whether switching the γ-globin CACCC box with the β-globin CACCC box would lead to the recruitment of EKLF to the γ-globin promoter in MEL cells (Fig. 3*C*). As shown in Fig. 3*D* (chimera 1), the exchange of the CACCC boxes did not result in EKLF recruitment to the γ-globin promoter. It is possible that the β-globin CACCC box alone may not be sufficient and that the promoter elements surrounding the β-globin CACCC box are also needed for EKLF recruitment. To address this possibility, we examined the recruitment of EKLF to chimera 2 (Fig. 3 *C* and *D*), in which the promoter elements upstream of the TATA box in the γ-globin promoter was replaced with the corresponding promoter elements from the β-globin promoter. EKLF was not recruited to chimera 2, suggesting either that the β-globin promoter elements upstream of the TATA box are not sufficient to recruit EKLF or that different β-globin promoter elements (e.g., the minimal promoter including the TATA box and initiator region) are required to recruit EKLF. To address the latter possibility, we examined the recruitment of EKLF to chimera 3, in which the minimal promoter region of the γ-globin promoter was exchanged with the corresponding minimal promoter region of the β-globin promoter. EKLF was also not recruited to chimera 3, suggesting that the complete β-globin promoter may be required for efficient recruitment of EKLF. In contrast, Sp1 was recruited to chimeras 1 through 3 (Fig. 3*D*). The cleavage sites are different for chimeras 1 through 3 because the distance between the CACCC box and the primer annealing sites vary for each chimera. These results point out how two related CACCC box-binding transcription factors Sp1 and EKLF have dramati-

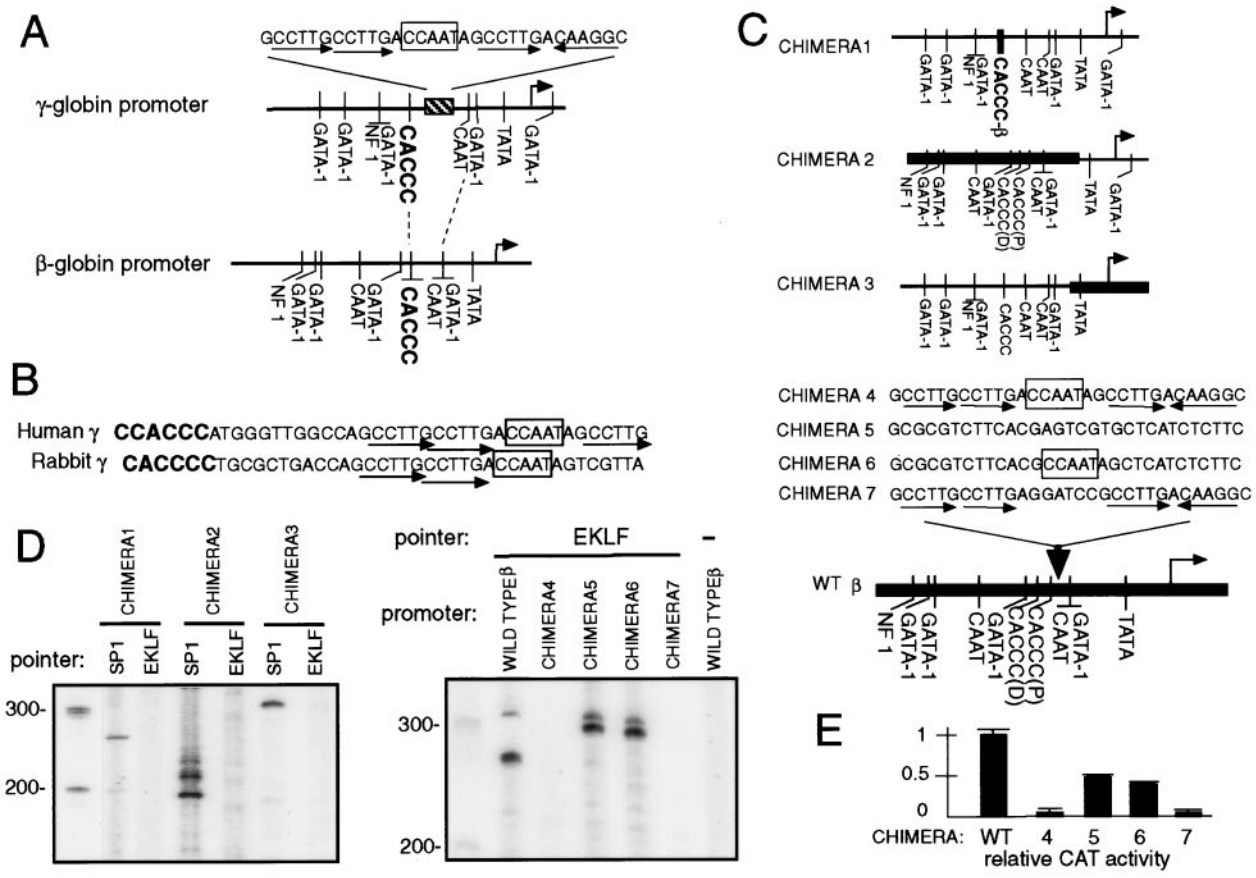

Fig. 3. Recruitment of EKLF to the γ-β chimeric promoters. (*A*) The human γ-globin promoter contains CCTTG repeats (striped box) in a region between the CACCC (bold letters) and the proximal CAAT (rectangle) boxes that is not present in the β-globin promoter. The position and orientation of each CCTTG repeat is marked with a horizontal arrow. The CACCC and CAAT boxes of the γ- and β-globin promoters are aligned with dashed lines. (*B*) The rabbit γ-globin promoter also contains the CCTTG repeat (horizontal arrow). (*C*) Diagram of γ-β chimeric promoters. In chimera 1, the CACCC box of the γ-globin promoter (thin line) was replaced with the CACCC box (CACCC-β) of the β-globin promoter. In chimera 2, the region upstream of the TATA box was replaced with the corresponding region of the β-globin promoter (thick line), and the minimal promoter region was from the γ-globin promoter (thin line). Chimera 3 is the opposite of chimera 2. In chimeras 4–7, the indicated sequences (31 bp) were inserted 3′ of the β-globin CACCC (proximal) box. (*D*) Recruitment of the EKLF pointer to the chimeric promoters in MEL cells. PIN*POINT assays were performed with EKLF pointer and a target plasmid containing the indicated chimeric promoter. (*Left*) The recruitment of Sp1 and EKLF pointer to chimeras 1 and 2 was detected by performing a primer extension with primer JS64 and the recruitment of Sp1 and EKLF pointer to chimera 3, with primer JS41. (*Right*) The recruitment of EKLF pointer to chimeras 4–7 was examined by performing a primer extension with primer JS41. The cleavage site in chimeras 5 and 6 were located ≈30 bp upstream of the cleavage site in the wild-type β-globin promoter because of the 31-bp insertion. (*E*) Suppression of EKLF pointer recruitment correlates with suppression of transcription. Chloramphenicol acetyltransferase (CAT) assays of the reporter constructs containing the γ-β chimera 4–7 were performed 48 hr after transiently transfecting them into MEL cells.

cally different requirements for recruitment. Furthermore, these results illustrate that the differences between the γ- and the β-globin promoter are not localized to a particular promoter element or a region but are scattered throughout the promoters.

To further explore the differences between the two promoters, we examined the possibility that the CCTTG repeat downstream of the CACCC box in the γ-globin promoter functions as a suppressor of EKLF recruitment. When the 31-bp fragment from the γ-globin promoter containing the CCTTG repeat was inserted downstream of the β-globin CACCC box, the recruitment of EKLF pointer to the β-globin CACCC box was suppressed (Fig. 3*D*, chimera 4). There was no suppression if a random 31-bp fragment was inserted (chimera 5). If a 31-bp fragment containing only the CAAT box was inserted, there was no suppression, suggesting that it is not the CAAT box binding factor that suppresses EKLF pointer recruitment (chimera 6). If a 31-bp fragment that contained the CCTTG repeat but not the CAAT box was inserted (chimera 7), the recruitment of EKLF pointer was suppressed, indicating that the CCTTG repeat is the suppressor of EKLF pointer recruitment. To determine whether EKLF recruitment to these chimeric promoters was related to the transcriptional activity of the respective promoters, chloramphenicol acetyltransferase assays were performed after transfecting these constructs (chimera 4–7) into MEL cells. As shown in Fig. 3*E*, the chimeric promoters 4 and 7, which failed to recruit EKLF, also had extremely low activity.

Although the CCTTG repeat suppresses the recruitment of EKLF, the observation that chimera 2, which is missing the CCTTG repeat, did not recruit EKLF indicates that it is not the sole determinant of EKLF recruitment. However, understanding how the γ-globin promoter is suppressed is important particularly because it has implications in the treatment of β-globin disorders. Therefore, we attempted to further characterize the suppressor activity of the CCTTG repeat. To determine whether the CCTTG repeat acts as a suppressor by binding to a trans-acting factor, EMSA (electrophoretic mobility shift assay) was performed with a radioactively labeled probe containing four CCTTG repeats by using nuclear extracts from MEL cells. We detected a bound complex that was easily competed with the unlabeled competitor (CCTTG, WTX4) but not with

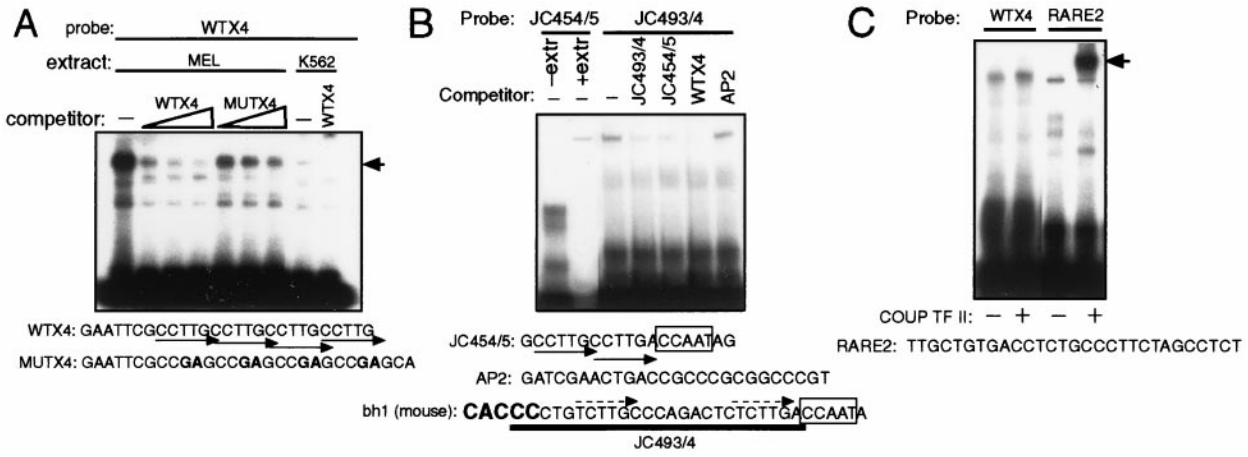

Fig. 4. A nuclear factor(s) binds to the CCTTG repeat. (*A*) Presence of CCTTG-specific factor in MEL cell nuclear extract. EMSA was performed with the MEL cell nuclear extract by using a probe containing four CCTTG repeats (WTX4). The shifted band is indicated with an arrowhead. Increasing amounts of unlabeled competitor probe WTX4 and a probe containing four mutated (CCGAG) repeats (MUTX4) at 10-, 50-, and 60-fold excess over the radioactively labeled probe were added to the binding mixture. The sequences of WTX4 and MUTX4 are shown below. (*B*) The repeat-binding sequence is conserved in mammalian fetal globin promoters. EMSA was performed with CCTTG containing probe JC454/5, which was derived from the γ-globin promoter, and JC493/4, which was derived from the sequence between the CACCC box and the CAAT box in the mouse bh1 promoter (shown below). Where indicated, 100-fold excess of the competitor was used. In the first lane, the MEL cell nuclear extract was left out of the binding reaction (−extr). AP2 binding sequence was used as negative control for competition. The TCTTG repeat in the bh1 promoter is marked with dashed arrows. (*C*) COUP TF II does not bind to the CCTTG repeat. EMSA was performed by using nuclear extract from COS-7 cells mock-transfected (−) or transfected with an expression vector for COUP TF II (+). WTX4 and RARE2 (sequence shown below), which contains binding sites for COUP TF II and related nuclear receptors, were used as probes. The COUP TF II complex is indicated with an arrowhead.

equivalent amounts of a mutant competitor (CCGAG, MUTX4) (Fig. 4*A*). This bound complex was also detectable with K562 cell extract but at a lower level (Fig. 4*A*; data not shown). We also examined whether the repeat was conserved in the promoter of the bh1 gene, the mouse equivalent of the γ-globin gene (23). Immediately downstream of the CACCC box in the promoter of the bh1 gene, there are two copies of TCTTG, which differs from CCTTG by a single C→T change. A probe containing the repeat (JC493/4) from the bh1 promoter bound to the same complex as the CCTTG repeat (Fig. 4*B*).

It has been reported that nuclear receptor COUP TF II (24) suppressed γ-globin promoter activity by interacting with a direct repeat element in the 31-bp suppressor region (25). The proposed COUP TF II binding site included the CAAT box but also overlapped the CCTTG sequence. We tested whether the factor that bound to the CCTTG repeat was COUP TF II by performing EMSA with the cellular extract derived from COS-7 cells transfected with a COUP TF II expression vector (Fig. 4*C*). Although COUP TF II bound to the probe RARE2, which contains binding sites for nuclear receptors such as COUP TF II, the CCTTG repeat did not bind to COUP TF II. We were also unable to supershift the CCTTG repeat binding protein with anti-COUP TF II antibody (data not shown). These results suggest that the CCTTG repeat-binding protein is not COUP TF II.

If the factor that is binding specifically to the CCTTG repeat is a suppressor, we may be able to squelch it by transfecting with a plasmid containing a high copy number of the repeats. We tested this by cotransfecting a luciferase reporter construct under the control of the wild-type γ-globin promoter (γ-luc) with a plasmid containing 15 copies of the CCTTG or CCGAG (mutant) repeat or an empty pBlueScript SK(+) vector. As shown in Fig. 5*A*, the plasmid containing the CCTTG repeat squelched the suppressor activity, but the plasmid with the CCGAG repeats or an empty pBlueScript SK(+) vector did not. When the repeat containing region was deleted from the γ-globin promoter (γΔ-luc), the expression of the γ-globin promoter was moderately increased, but it was not affected by either competitor plasmids.

To test whether the CCTTG repeats can suppress a heterologous promoter, we inserted four direct CCTTG repeats upstream of the enhancerless SV40 promoter [SV40(4R)-luc] and measured the promoter activity in K562 (shown) and MEL cells (data not shown). The activity of SV40(4R)-luc reporter construct was three-fold lower than the SV40 reporter construct without the repeats (SV40-luc) in both cell types (Fig. 5*B*). As was the case with the γ-globin promoter, squelching the suppressor protein with the competitor plasmid containing the

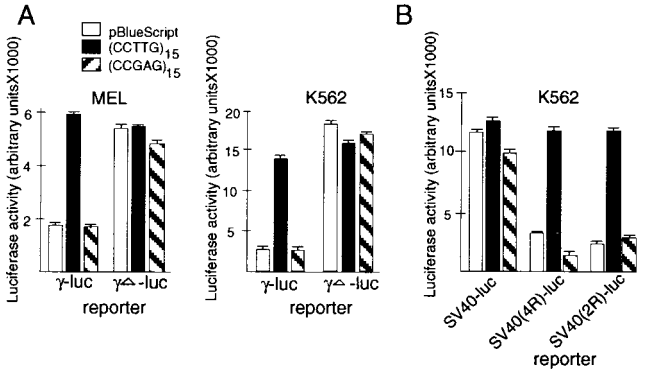

Fig. 5. Squelching of the suppressor protein by competing with the CCTTG repeat. (*A*) A luciferase reporter construct containing the wild-type γ-globin promoter (γ-luc) or a luciferase reporter construct containing the γ-globin promoter with the 31-bp suppressor binding region deleted (γΔ-luc) was cotransfected with either empty pBlueScript SK(+) (Stratagene) vector (open bar), a plasmid containing 15 copies of CCTTG repeat (solid bar), or a plasmid containing 15 copies of CCGAG mutated repeat (striped bar). Luciferase assays were performed 48 hr after transfection into MEL (*Left*) or K562 cells (*Right*). (*B*) A luciferase reporter construct containing the enhancerless SV40 promoter (SV40-luc) or a reporter construct containing the SV40 promoter with four copies of the CCTTG repeats inserted upstream [SV40(4R)-luc] or two copies of the CCTTG repeats [SV40(2R)-luc] was cotransfected with the competitor plasmids into K562 cells. Luciferase assays were performed as described in *A*. Results with MEL cells were very similar (data not shown).

CCTTG repeats derepressed the SV40(4R)-luc reporter construct. The squelching of the suppressor protein did not occur with the competitor plasmid containing the CCGAG repeat or an empty pBlueScript SK(+) vector. Because the rabbit γ-globin promoter contained two copies of the CCTTG repeat instead of four, we also tested whether two copies of the repeat [SV40(2R)-luc] were sufficient for suppression. Like SV40(4R)-luc, the activity of the SV40(2R)-luc reporter construct was suppressed in comparison to SV40-luc and was derepressed with the competitor plasmid. Because the CCTTG repeats did not block Sp1 recruitment to the γ-globin promoter (see Fig. 2*A*), it is likely that the repeats do not suppress the SV40 promoter by blocking Sp1 recruitment. However, we cannot rule out the possibility that the repeats block Sp1 recruitment but very weakly, such that it cannot be detected by PIN*POINT.

Discussion

The role of EKLF and Sp1 on the activity of the γ- and β-globin promoters have been studied previously by using as a readout the activity of the reporter constructs (14, 15, 26–28). However, transcription level is determined by a large number of events, including recruitment of individual transcription factors and basal transcriptional machinery, mRNA processing, as well as a modification of RNA polymerase II, which regulates processivity of the polymerase (reviewed in refs. 29 and 30). In this report, we defined the differences between the γ- and β-globin promoters by examining their ability to recruit EKLF. To do this, we took advantage of the unique ability of PIN*POINT to visualize protein-DNA interaction *in vivo*. Our findings indicate that the recruitment of EKLF depends on its interaction with transcription factors scattered throughout the β-globin promoter. Although the transcription factor binding sites are somewhat similar between the γ- and β-globin promoters, only the β-globin promoter appears to possess the proper arrangement of transcription factor binding sites for EKLF recruitment. The identity of all of the transcription factors and the particular arrangement of their binding sites in the β-globin promoter that makes it the preferred target over the γ-globin promoter for EKLF is unknown. However, we have determined that one of the reasons EKLF is not recruited to the γ-globin promoter is that it contains four repeats of CCTTG immediately downstream of its CACCC box, but the β-globin promoter does not. The trans-acting factor that binds to the repeat has a moderate suppressor activity on the γ-globin promoter as well as on a heterologous promoter that contains the repeat.

Because K562 cells express γ-globin and contain low levels of the CCTTG-binding activity, it is puzzling that the effect of squelching was equal to, if not more than, squelching in MEL cells (Fig. 5*A*). We do not have a good explanation for this. However, the transfection efficiency and probably the number of plasmid molecules entering the cell are very different between these two cell lines (data not shown); comparing the squelching effect between two cell lines with such differences may not be meaningful.

Increased levels of γ-globin in HPFH (hereditary persistence of fetal hemoglobinemia) can ameliorate the clinical course of inherited β-globin disorders such as sickle cell anemia and β^+ thalassemia (31, 32). It is intriguing that the Greek type HPFH has a G-to-A mutation in one of the CCTTG repeats (31). However, we have not been able to demonstrate that the Greek type mutation affects the CCTTG repeat function. Despite this, our results may have clinical implications. Inactivation of the γ-globin suppressor protein and the resulting increase in γ-globin may partially improve the clinical course of β-globin disorders. As such, the CCTTG repeat-binding protein may be a potential drug target for treatment of sickle cell anemia and β thalassemia.

We are grateful to Dr. James Bieker for EKLF cDNA and MEL cells, Paul Ney for pNL6, Ming Jer Tsai for COUP TF II cDNA, and the members of the laboratory of Dr. Jay Chung for reviewing this manuscript.

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