Role of erythroid Kruppel-like factor in human γ- to β-globin gene switching

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Erythroid Kruppel-like factor (EKLF) is an erythroid-specific transcription factor that contains zinc finger domains similar to the Kruppel protein of Drosophila melanogaster. Previous studies demonstrated that EKLF binds to the CACCC box in the human β-globin gene promoter and activates transcription. CACCC box mutations that cause severe β-thalassemias in humans inhibit EKLF binding. Results described in this paper suggest that EKLF functions predominately in adult erythroid tissue. The EKLF gene is expressed at a 3-fold higher level in adult erythroid tissue than in fetal erythroid tissue, and the EKLF protein binds to the human β-globin promoter 8-fold more efficiently than to the human γ-globin promoter. Co-transfection experiments in the human fetal-like erythroleukemia cell line K562 demonstrate that over-expression of EKLF activates a β-globin reporter construct 1000-fold; a linked γ-globin reporter is activated only 3-fold. Mutation of the β-globin CACCC box severely inhibits activation. These results demonstrate that EKLF is a developmental stage-enriched protein that preferentially activates human β-globin gene expression. The data strongly suggest that EKLF is an important factor involved in human γ to β-globin gene switching.

All vertebrate animals switch hemoglobins during development. In humans, the first site of erythropoiesis is the yolk sac blood islands. Erythroid cells in the yolk sac are formed from embryonic mesoderm at approximately 3 weeks of gestation. The first hemoglobin produced by these cells are tetramers composed of 2 γ-globin or 2 α-globin polypeptides and 2 ε-globin polypeptides. The hemoglobin genes are designated Gower I (α2ε2) and Gower II (α2ε2). γ-Globin gene expression gradually decreases, and α-globin expression gradually increases over the next few weeks until Gower II (α2ε2) is the predominate hemoglobin. At approximately 5 weeks of development, hematopoietic stem cells from the yolk sac migrate to the fetal liver and initiate erythropoiesis in this organ. Fetal liver then becomes the major site of erythropoiesis, and there is a concomitant switch in hemoglobin production; ε-Globin gene expression decreases, and the fetal γ-globin gene is activated. The major hemoglobin produced at this stage of development is, therefore, fetal hemoglobin (α2γ2). Finally, hematopoietic stem cells from the fetal liver migrate to the bone marrow. This organ then becomes the major site of erythropoiesis, and there is a final switch in hemoglobin synthesis. Expression of the γ-globin gene steadily decreases, and expression of the δ- and β-globin genes gradually increase until approximately 98% of total hemoglobin is hemoglobin A (α2β2) and approximately 1% is hemoglobin A2 (α2δ2); the δ-globin promoter is enfeebled and, therefore, is transcribed poorly. The γ-globin gene continues to be transcribed in a minor population of erythroid cells that develop in the adult bone marrow; these cells are designated F cells, and the HbF in these cells accounts for approximately 1% of total hemoglobin in adult blood (1).

The molecular mechanisms that direct hemoglobin switching during development are almost completely unknown. In humans, a powerful regulatory sequence designated the locus control region (LCR) is located far upstream of the β-globin locus on chromosome 11 (for review, see Refs. 2–5)). The β-globin LCR has two important functions. First, these sequences open a chromosomal domain that extends over 200 kb. This chromatin decondensation renders individual genes in the locus more accessible to transcription factors that control temporal specific expression. Secondly, the LCR acts as a master enhancer; individual globin gene family members compete for interactions with the LCR to determine which genes are expressed at specific developmental stages. Positive and negative regulatory factors that bind to specific globin gene promoters and proximal enhancers or silencers provide individual genes with a competitive advantage or disadvantage for interaction with the LCR. Once interactions between a particular globin gene(s) and the LCR is established, the complex is relatively stable and commits this chromosomal allele(s) to express ε, γγ, and Aγ or δ- and β-globin throughout the lifetime of the cell.

Although developmental stage-specific factors have been proposed to regulate globin gene expression during development, no proteins that control human γ to β-globin gene switching have been identified. In this paper, we have examined the role of erythroid Kruppel-like factor (EKLF) in globin gene switching. EKLF is an erythroid cell-specific transcriptional activator that contains three zinc fingers homologous to the Kruppel family of transcription factors (6). As shown by crystallographic and “finger-swapping” experiments with other members of this family, each finger contacts 3 base pairs, such that the binding site for the EKLF protein was predicted to be 3'-GGNGGNN-5'. Based on this information, it was dem-

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The abbreviations used are: LCR, locus control region; EKLF, Erythroid Kruppel-like factor; kb, kilobase(s); Luc, luciferase; CAT, chloramphenicol acetyltransferase; γLuc, γ-globin/Luc; β-CAT, β-globin/CAT; HS 2, hypersensitive site 2.
onstrated that EKLF binds to and mediates its transcriptional activation via the human and murine adult β-globin CAC site (5′-CCACACCCCT-3′) (6), a site known to be critical for β-globin expression (7–10). Methylation interference studies indicated that EKLF forms close contacts to each of the guanine residues within this extended 9-base pair site, such that point mutation of some of these residues, including those that give rise to β-thalassemia, drastically disrupt binding (11). These results suggest that EKLF may be intimately involved in the regulation of globin expression through its interaction with the CACC element.

However, inspection of CAC-related sequences in other murine and human β-globin promoters in the context of the complete 9-base pair EKLF-binding element reveals that these sites do not form a homogeneous group. Of particular interest for the present study is the sequence 5′-CTCCACCCCA-3′ in the human fetal Aγ- and Gγ-globin promoter (and in the murine embryonic γ-globin promoter). This sequence contains a mismatch to the predicted EKLF binding site, i.e., binding by the EKLF amino-terminal fragment to 5′-CCN-3′. Our previous studies have emphasized the importance of the interactions between nucleotides in the complete β-CAC site with critical EKLF amino acid residues (6, 11). A decrease in EKLF binding affinity to the variant γ-CAC site could play a major role in determining the relative levels of γ- and β-globin transcription. We have therefore directly tested the ability of EKLF to bind to the γ-globin CAC site in vitro and have examined the role of EKLF in human γ- to β-globin gene switching in vivo.

MATERIALS AND METHODS

Competitive Gel Shift Assays—The competitive gel shift assays were performed as described (6, 11). The presence of a doublet in the gel shift experiment results from the production of different site glutathione S-transferase-EKLF fusion proteins in the preparation as described by Miller and Bieker (6).

Northern Blot Hybridizations—Northern blot hybridizations were performed as previously described (12). The EKLF probe was a 0.74-kb SnaBI-PvuI fragment of the EKLF cDNA; this probe lacks the zinc finger coding region. The mouse α-globin probe was a 1.1-kb ApcI-XbaI fragment, which contains the entire enigmatic clone.

Plasmid Constructions—The construction of the HS 2-yluciferase reporter has been described (12). HS 2-β-luciferase was constructed similarly to HS 2-yluciferase. A SnaBI-NcoI fragment that contained human β-globin promoter and 5′-untranslated sequences from −265 to −48 was blunt-ended with S1 nuclease and inserted into a blunt-ended BglII site between HS 2 and luciferase in the HS 2-yluciferase plasmid (12).

HS 2-yluciferase-β/CAT was constructed in several steps. β/CAT was made by inserting the blunt-ended β-globin promoter fragment described above into the plasmid pCAT-Basic (Promega), which had been cut with XbaI and blunt-ended with Klenow polymerase. The following three fragments were then ligated to make HS 2-yluciferase-β/CAT: a 4.5-kb KpnI-SalI fragment from HS 2-yluciferase containing HS 2, the γ-globin promoter, the luciferase gene, and the SV40 splice and polyadenylation signals; a 2.9-kb SfiI-BamHI β/CAT fragment containing the β-promoter, CAT gene, and splice and poly(A) signals; and a 2.9-kb BamHI-KpnI fragment from pGL2-Basic (Promega) containing prokaryotic vector sequences.

To construct HS 2 (CACC-yluciferase-β/CAT, a mutant HS 2 fragment containing the scrambled CACC motif was derived from a previously described plasmid (13) (plasmid 5′-HS 2 (K-P) β 8669–386a). The 1.4-kb KpnI-SnaI HS 2 fragment from this mutant plasmid was used to replace the corresponding wild type region in HS 2-yluciferase-β/CAT.

To make HS 2-yluciferase-β (−87)/CAT, a β-promoter containing the −87 to +48 region of C β-thalassemia mutation was constructed using the mega-primer mutagenesis method (14–16). The outer primers overlapped the SnaBI site at −265 and the NcoI site at +48 of the human β-promoter. The 3′-primer changed the NcoI site to a SnaBI site (underlined in the sequence below) so that a blunt end promoter fragment could be easily excised. The 5′-primer was a linearized HindIII subclone of the human β-promoter in pUC 19. Primers used to amplify the mutant promoter were 1) the upstream pUC reverse primer from New England Biolabs, 2) the mutagenic oligonucleotide 5′-CTCGGGAGTAGATGGTGAGGTCGTCCACGCCT-3′ (the mutated base is underlined), and 3) the downstream oligonucleotide 5′-AGGGTGACCTAGTATCGTGGGTCCTAAGT-3′ (the underlined bases represent a SnaBI site). The resulting SnaBI fragment containing the β (−87) mutation was used to make HS 2-yluciferase-β (−87)/CAT as described for the wild type plasmid. All promoter sequences were verified by dideoxy sequencing (17) using the Sequenase kit (U. S. Biochemicals Corp.).

Transactivation Analysis—Co-transfections and reporter gene assays were performed as described (12), except that luciferase assays were measured on a Turner model 20 luminometer (Promega) using the Promega Luciferase Assay System. Extracts were diluted 1:10 for luciferase assays (20 μl of diluted extract assayed for all samples), and representative values were in the range of 600 light units/μl of extract/β-galactosidase A420 for vector controls and 1,800 light units/μl of extract/β-galactosidase A420 for EKLF co-transfections (linear range, 0.1–10,000). For CAT assays, vector control extracts were assayed undiluted (50 μl of extract), and EKLF co-transfection extracts were diluted up to 200-fold for assays. For the wild type HS 2-yluciferase-β/CAT reporter, representative values obtained were on the order of 50 cpm/μl of extract/β-galactosidase A420 in vector controls and about 50,000 cpm/μl of extract/β-galactosidase A420 for EKLF co-transfections (minimum detectable activity was 10 cpm/μl of extract/β-galactosidase A420 or 5-fold over background under these assay conditions).

RESULTS

Fig. 1A illustrates a competitive gel shift experiment designed to measure the relative binding efficiency of EKLF to the CACC boxes in the human β- and γ-globin gene promoters. A double-stranded oligonucleotide containing the β-globin CACC box was end-labeled and incubated with purified EKLF (11) in the presence of increasing amounts of unlabeled β- and γ-globin CAC box oligonucleotides. The results were quantitated and graphed as illustrated in Fig. 1B. Under the conditions of the assay, an 11-fold excess of β-globin CAC site was required to inhibit the EKLF-CAC shift by 50%; however, a 90-fold excess of the γ-globin CAC site was required for 50% inhibition. These results demonstrate that EKLF binds approximately 8-fold more efficiently to the β-globin CACCC box than to the γ-globin CACC box; this is consistent with the very weak EKLF/γ-CAC gel shift that is observed relative to that seen with EKLF/β-CAC (Fig. 1C). The higher binding affinity of EKLF to adult versus fetal globin gene promoters suggests that EKLF may be involved in γ- to β-globin gene switching.

The level of EKLF expression in fetal and adult erythroid tissue was also examined. Fig. 2 illustrates a Northern blot of mouse yolk sac, fetal liver, and reticulocyte RNA probed with the murine EKLF cDNA clone. The filter was subsequently stripped and reprobed with a mouse α-globin clone as a control. Bands were quantitated on a phosphorimager, and EKLF expression was normalized to α-globin expression. The results demonstrate that EKLF expression in mouse fetal liver, which is an adult erythroid tissue, is 3-fold higher than expression in mouse yolk sac. The switch from embryonic/fetal globin to adult globin expression in the mouse occurs when the site of erythropoiesis shifts from yolk sac to fetal liver at approximately 14 days of development; adult globin expression is then maintained when bone marrow becomes the major site of erythropoiesis at birth. The higher levels of EKLF mRNA in adult compared with fetal tissue also suggest that EKLF may be involved in γ- to β-globin switching.

To determine the functional consequences of differential EKLF binding to human γ- and β-globin gene promoters, co-transfection experiments in K562 cells were performed. These cells normally synthesize little EKLF (Fig. 2), and no β-globin mRNA can be detected (data not shown). γ-Globin/luciferase (γ/Luc) and β-globin/CAT (β/CAT) reporter genes were inserted downstream of the LCR HS 2 (18) (Fig. 3A), and these constructs were co-transfected with an EKLF expression vector into K562 cells. Fig. 3B demonstrates that EKLF stimulates γ/Luc expression only 3-fold; how-
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**Fig. 1.** Competitive gel retardation analysis of EKLF binding to variant CAC site-containing oligonucleotides *in vitro*. Gel shift assays used radiolabeled adult β-globin oligonucleotides and the indicated non-radioactive competitor oligonucleotides at 0-, 20-, 50-, 100-, 200-, and 400-fold molar excess (lanes 2–7, respectively). Lane 1 contained no protein added to the incubation. Data for two preparations of purified EKLF are shown to demonstrate the reproducibility of the assay. The autoradiograph of the gel resulting from all the assays is shown in A. The amount of shift seen without any competitor is defined as 100%. The point at which each of these curves crosses the “50% signal remaining” line was used as the basis for estimating the competitive ability of each oligonucleotide for binding to EKLF relative to adult β-globin CAC. C, direct binding analyses of EKLF and radiolabeled β- or γ-CAC site-containing oligonucleotides *in vitro* are shown. The specific activities of these probes were equivalent, and equal counts/min were loaded in each lane.

**Fig. 2.** Northern blot analysis of EKLF and mouse α-globin expression. Lanes 1–6 were loaded with 2 µg of total RNA from the indicated cell lines and tissues. Lane 1, human erythroleukemia cells (K562); lane 2, mouse erythroleukemia cells uninduced (MEL-U); lane 3, mouse erythroleukemia cells induced with 1.5% dimethyl sulfoxide for 3 days (MEL-I); lane 4, yolk sacs dissected from 10.5-day-old mouse embryos (10.5 d YS); lane 5, fetal liver dissected from 16-day-old mouse fetuses (16 d FL); lane 6, adult blood from phenylhydrazine-treated mice (Ad. Blood). Phosphorimager quantitation of bands (Molecular Dynamics Phosphorimager) shows that the level of EKLF message/α-globin message is 3-fold higher in 16 d FL than in 10.5 d YS. A faint band of EKLF and α-globin mRNA is observed in the K562 lane after longer exposure (data not shown).

**Fig. 3.** Western blot analysis of EKLF and α-globin expression. Lanes 1–6 were loaded with 3 µg of total protein from the indicated cell lines and tissues. Lane 1, human erythroleukemia cells (K562); lane 2, mouse erythroleukemia cells uninduced (MEL-U); lane 3, mouse erythroleukemia cells induced (MEL-I); lane 4, yolk sacs dissected from 10.5-day-old mouse embryos (10.5 d YS); lane 5, fetal liver dissected from 16-day-old mouse fetuses (16 d FL); lane 6, adult blood from phenylhydrazine-treated mice (Ad. Blood). As described above, a mutation at −87 (CACCC to CACC) in the β-globin promoter inhibits β-globin gene expression and causes β-thalassemia in humans (19, 20). This −87 mutation was introduced into the β-globin gene promoter in the linked γ/Luc-β/CAT reporter construct, and the plasmid was co-transfected with the EKLF expression vector into K562 cells. The data in Fig. 5 demonstrate that the −87 mutation strongly inhibits EKLF activation of the β-globin gene (1000 to 4-fold activation). Mutation of the phylogenetically conserved CACCC box (21) located approximately 15 base pairs downstream of the AP1-like sites in HS 2 also decreases β-globin gene activation (1000 to 730-fold activation).
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Models of human globin gene switching postulate that developmental stage-specific transcription factors bind to promoters and proximal enhancers or silencers and influence the interaction of \( \epsilon \), \( \gamma \), and \( \beta \)-globin genes with the powerful LCR. Although these stage-specific proteins have been postulated for many years, no positive or negative regulatory factors that direct \( \gamma \) to \( \beta \)-globin gene switching have been identified. The data described above strongly suggest that EKLF is a developmental stage-enriched factor that is involved in the switch from human \( \gamma \)-globin to \( \beta \)-globin gene expression. The binding affinity of EKLF is 8-fold higher for the \( \beta \)-globin promoter than for the \( \gamma \)-globin promoter (Fig. 1), and the EKLF gene is expressed at a 3-fold higher level in adult erythroid tissue than in fetal erythroid tissue (Fig. 2). Although we have not yet quantitated EKLF protein in these cells, Northern blot data (Fig. 2) suggest that EKLF levels in adult erythroid tissue are significantly higher than in fetal erythroid tissue. To determine the functional consequences of differential EKLF concentration and binding affinity, we co-transfected an EKLF expression vector with HS 2 \( \gamma \)-Luc and HS 2 \( \beta \)-CAT reporter constructs into K562 cells. These feto-like erythroleukemia cells express \( \gamma \)-globin and normally synthesize little EKLF but not \( \beta \)-globin genes and normally synthesize little EKLF (Fig. 2). After transfection, \( \gamma \)-Luc was activated only 3-fold, but \( \beta \)-CAT was activated 30-fold (Fig. 3). When \( \gamma \)-Luc and \( \beta \)-CAT were linked in the same construct (HS 2 \( \gamma \)-Luc-\( \beta \)-CAT) and co-transfected with the EKLF expression vector into K562 cells, the \( \beta \)-globin promoter was activated 1000-fold; the \( \gamma \)-globin promoter was activated 30-fold. EKLF stimulated \( \beta \)-CAT activity 1050-fold and \( \gamma \)-Luc activity only 3-fold. A direct comparison of \( \beta \)-CAT activities from HS 2 \( \gamma \)-Luc-\( \beta \)-CAT transfections and HS 2 \( \beta \)-CAT transfections were also made (data not shown). In the absence of exogenous EKLF, \( \beta \)-CAT activity from the HS 2 \( \gamma \)-Luc-\( \beta \)-CAT reporter was 120-fold lower than \( \beta \)-CAT activity from the HS 2 \( \beta \)-CAT reporter.
bin promoter was activated only 3-fold (Fig. 4). Mutation of the β-globin CACCC box at −87 (CACCC to CAGCC) strongly inhibited EKLF activation (1000 to 4-fold activation Fig. 5). These results suggest that EKLF is an important factor in human γ- to β-globin gene switching and that the CACCC boxes are critical elements in this switch. Mutation of the phylogenetically conserved CACCC site in HS 2 modestly inhibits EKLF activation in transient assays (Fig. 5). Analysis of this same mutation in transgenic mice shows a similar reduction in activity (13). Reddy et al. (22) recently showed that the HS 2 CACCC site is footprinted in vivo in adult human erythroblast but not in the fetal environment of K562 cells. These results, together with the results presented in this paper, suggest that the HS 2 CAC site may also contribute to β-globin gene activation.

The lower binding affinity of EKLF for the γ-globin CAC site is not entirely unexpected. Methylation interference demonstrates that EKLF interacts with all the guanine residues on the G-rich strand of the β-globin CAC site, including the sequence 5′-GGN-5′, which is the putative first finger target site (11). These studies showed that changes of single guanine residues had a dramatic effect on EKLF binding affinity to those variant sites. In the γ-CAC site, the first finger target site would be 3′-GAG-5′, yielding a loss of an important guanine residue. The lower affinity for this site indicates that binding of the amino-terminal EKLF zinc finger is an important contributor to the overall affinity of EKLF-CAC site interaction. As a result, efficient EKLF binding to the CTCCACCCA site present in the γ-globin promoter is very low and may be heavily dependent on the effective EKLF protein concentration or on the presence of a cofactor. Alternatively, Ikuta and Kan (23) have demonstrated an in vivo footprint on the γ-CAC box but not on the β-CAC box in K562; therefore, transcription of the γ-globin gene may require another CACCC element-binding factor that is primarily active in fetal, rather than adult, erythroid cells.

Competition models of globin gene switching predict that enhanced interaction of one globin gene with the LCR necessarily decreases the interaction of another gene with the LCR. This mechanism accounts for the precise developmental specificity of globin gene expression. However, in the experiments described above, both γ- and β-globin genes were stimulated. EKLF expression enhanced βCAT activity 1000-fold, and γLuc activity did not decrease but increased 3-fold. Stimulation of both genes in this instance may occur because all of the regulatory factors required for expression of γ- and β-globin genes are present at the same time. K562 cells normally contain the factors necessary for γ-globin expression, and ectopic expression of EKLF apparently provides an additional factor necessary for β-globin gene activation. Expression of both genes in the same cell would be the predicted result if the equilibrium constants for LCR-γ and LCR-β interactions are equivalent when both fetal and adult regulatory factors are present.

If EKLF was the only positive factor necessary for γ- to β-globin gene switching, one would predict that overexpression of this factor in fetal erythroid cells would activate the endogenous β-globin gene. However, we stably transformed K562 cells with the EKLF expression vector, and no endogenous β-globin mRNA was detected. This result suggests that additional factors are required to activate a chromosomal copy of the adult gene. Although the entire β-globin locus appears to be in an "open" or DNase I-sensitive domain in erythroid cells, local changes in chromatin structure around individual genes may play a role in switching. Perhaps additional temporal specific factors are required to reposition nucleosomes so that the CACCC boxes are more or less accessible to EKLF (24–26).

Jane et al. (27) recently defined a stage-selector element in the γ-globin promoter that appears to be important in γ-globin gene activation. This sequence is located between −54 and −35, and insertion of the stage-selector element in a β-globin gene construct results in a 10-fold increase in β-globin gene expression in K562 cells. A fetal-specific protein complex designated SSP (stage-selector protein) binds to the sequence (28) and is most likely involved in γ-globin gene activation in fetal development. Therefore, this protein and EKLF may be critical fetus- and adult-specific proteins that are responsible for human ε- to γ-globin and γ- to β-globin gene switching during development.

The results described above strongly suggest that EKLF is an important factor in temporal control. Targeted mutation of the EKLF gene in embryonic stem cells should provide additional information on the role of EKLF in hemoglobin switching. Based on the data in this paper, the phenotype of mice that are homozygous for a mutation in EKLF can be predicted. These mice should survive through early development but then die between 12 and 14 days of gestation when the switch from fetal to adult globin gene expression occurs.

REFERENCES