Differential Binding of Erythroid Kruppel-like Factor to Embryonic/Fetal Globin Gene Promoters during Development*

Received for publication, February 7, 2006. Published, JBC Papers in Press, April 10, 2006.

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The competition model for β-like globin gene switching during development predicts that differential binding of transcription factors to globin gene promoters and/or proximal enhancers regulate the competitive interactions of globin gene family members with the powerful locus control region (LCR). Direct interactions of individual genes with the LCR are essential for high level expression in erythroid cells. In this paper, we have demonstrated, by chromatin immunoprecipitation, that erythroid-Krupple-like factor (EKLF) binds to embryonic/fetal globin gene promoters in primitive (but not in definitive) erythroid cells. EKLF binds strongly to adult globin gene promoters and to LCR sequences HS4, HS3, HS2, and HS1 in both primitive and definitive erythroid cells. Trimethylation of histone H3K4 and H3K27 at the embryonic/fetal and adult globin gene promoters is equivalent in definitive cells; therefore, the differential binding of EKLF to these promoters does not appear to result from changes in chromatin configuration. Interestingly, the level of EKLF in definitive cells is 3-fold higher than the level in primitive cells. These results suggest that temporal-specific changes in EKLF abundance result in differential binding of this essential erythroid transcription factor to embryonic/fetal globin gene promoters during development and that these changes in EKLF binding specificity mediate the competitive interactions of globin gene family members with the LCR.

The genes encoding β-like subunits of mammalian hemoglobins are expressed in a precise tissue- and developmental stage-specific pattern (1). Although erythroid-specific expression of human ε-, γ-, and β-globin genes in the yolk sac, fetal liver, and bone marrow has been known for decades, the precise molecular mechanisms that direct this exquisite regulation are still unknown. The ε-, γ-, and β-globin genes are linked directly to the LCR (2–7). These results strongly suggested that human γ-globin gene sequences are essential for correct human β-globin gene developmental control. Later experiments demonstrated that disruption of the γ-globin promoter CACCC box results in the loss of β-globin gene developmental specificity in transgenic mice (9, 10). Therefore, the γ-globin gene CACCC box appears to be essential for correct globin gene switching.

In this paper, we have demonstrated, by chromatin immunoprecipitation (ChIP), that EKLF binds to embryonic/fetal globin gene promoters in primitive (but not definitive) erythroid cells. Second, we have found that EKLF binds strongly to adult globin gene promoters in both primitive and definitive cells. Finally, we have demonstrated that EKLF concentrations increase ~3-fold from primitive to definitive erythroid cells. We propose that this temporal-specific change in EKLF abundance is responsible for differential binding of EKLF to the embryonic/fetal globin gene promoters and, therefore, is critical for globin gene switching. Previously published functional data are consistent with this model. Underexpression of EKLF in heterozygous EKLF knock-out mice results in delayed switching (15, 16), and overexpression of EKLF in transgenic mice results in premature globin gene switching (17).

**EXPERIMENTAL PROCEDURES**

*Knock-in Mouse Lines and Antibodies—Generation of homozygous hemagglutinin/erythroid Kruppel-like factor/tandem affinity purification (HA/EKLF/TAP) knock-in mice was reported previously (18). The mice used for ChIP analysis in Figs. 1 and 2 were also homozygous for the HbβS globin haplotype. The ΔLCR mice used for ChIP analysis in...
Fig. 4 were produced by removing HS1–4 in an 18- kb deletion on the HbbD background. ΔLCR mice were bred with HA/EKLF/TAP knock-in mice, and the offspring were mated to generate ΔLCR mutant mice (ΔLCR (D)/WT (S)) and wild-type mice (WT (D)/WT (S)); the ΔLCR and WT mice were also homozygous for HA/EKLF/TAP. Normal rabbit IgG (catalog number sc-2027), anti-HA antibody (catalog number sc-7392), and anti-GFP antibody (catalog number sc-9996) were obtained from Santa Cruz Biotechnology. Anti-trimethyl-histone H3 K4-(07-473) and anti-trimethyl-histone H3 K27 (07-449, 05-851) were purchased from Upstate Biotechnology. Anti-trimethyl-histone H3 K9 (catalog number ab8898) was obtained from Abcam.

Reverse Transcription-PCR of Yolk Sac and Fetal Liver RNA—Total RNA was purified from a 10.5-dpc yolk sac and a 14.5-dpc fetal liver using TRIzol LS reagent (Invitrogen) and reverse transcribed with SuperScript (Invitrogen) using random hexamers as primers. Duplex PCR was performed to determine the relative abundance of ey2 and βg transcripts. The ey2 and βg primer sequences are listed in the supplemental information.

Chromatin Immunoprecipitation Assays—Chromatin immunoprecipitations were performed as described previously (19), except for the following modification. To prevent protein A of the TAP tag from binding to the antibody, anti-HA and anti-GFP-agarose were blocked with a Molecular Dynamics phosphorimaging device. The enrichments of ey2 and βg transcripts. The ey2 and βg primer sequences are listed in the supplemental information.

Western Blot Analysis—Whole cell extracts were produced from Ter119+ cells purified from a 10.5-dpc yolk sac and from 14.5-dpc fetal liver cells. Proteins were separated on 10% SDS-polyacrylamide gels and transferred to a nitrocellulose filter (Schleicher & Schuell). Anti-HA antibody (horseradish peroxidase-conjugated; Abcam) was used to detect HA-tagged EKLF protein, and anti-GAPDH antibody (horseradish peroxidase-conjugated; Abcam) was used to detect GAPDH, which served as a loading control. Western blot bands were quantitated by densitometry (Bio-Rad), and HA/EKLF/TAP signals were normalized to GAPDH.

RESULTS

EKLF Binds to ey2 and βh1 Promoters in Primitive (but Not Definitive) Erythroid Cells— Although much has been learned about EKLF function since Miller and Bieker (21) discovered this erythroid-specific protein in 1993, large gaps still exist in our understanding of the way in which EKLF regulates globin gene expression in vivo. One problem has been the difficulty of producing specific and sensitive EKLF antibodies that can be used in ChIP experiments to assess EKLF binding to LCR and β-like globin genes in primary primitive and definitive erythroid cells. To overcome this obstacle, we replaced the endogenous murine EKLF gene with a HA- and TAP-tagged version of the gene in embryonic stem cells and produced mice that are homozygous for the modified allele (18). These mice were not anemic, and globin gene switching during development was normal compared with wild-type controls (Fig. 1A). Therefore, the HA/EKLF/TAP knock-in mice could be used to define the developmental pattern of EKLF binding to globin locus regulatory sequences by ChIP experiments with the highly specific and sensitive anti-HA antibody.

Fig. 1B illustrates the mouse β-globin locus and the location of primers used for PCR of immunoprecipitated chromatin DNA. Panels C–F illustrate ChIP results from bone marrow, fetal liver, and yolk sac cells of homozygous HA/EKLF/TAP mice. Fig. 1C is a control and demonstrates that no enrichment of globin sequences was detected in anti-HA (compared with anti-GFP)-precipitated chromatin from Ter119− bone marrow cells of HA/EKLF/TAP mice. The data in Fig. 1D demonstrate a dramatic enrichment of HS4, HS3, HS2, HS1, and βg promoter sequences in anti-HA-precipitated chromatin from Ter119+ bone marrow cells of HA/EKLF/TAP mice. These results strongly suggest that EKLF binds to these sequences in definitive erythroid cells in vivo. Interestingly, the data also demonstrate that EKLF does not bind to H55 or to the ey2 and βh1 genes in definitive erythroid cells. Finally, the specificity of the assay was demonstrated by the absence of enrichment for sequences between HS2 and HS3 in HA-immunoprecipitated chromatin.

These data are similar to the results recently reported by Im et al. (22) on EKLF binding to β-globin locus regions in G1E cells (23). Im et al. demonstrate EKLF binding to HS3, HS2, H55, and to the βm1-globin gene promoter in this definitive erythroid cell line. Interestingly, EKLF binding to HS4 was not observed in G1E cells. EKLF binding to HS4 in Ter119+ bone marrow cells (see previous paragraph) (but not in G1E cells) may reflect differences in the cell types analyzed or in the antibodies utilized for ChIP.

As expected, the same EKLF binding pattern that was observed in Ter119+ bone marrow cells was also observed in 14.5-dpc fetal liver cells (Fig. 1F). EKLF bound strongly to HS4, HS3, HS2, H55, and βg promoter sequences in anti-HA-precipitated chromatin from HA/EKLF/TAP fetuses, and no binding was observed at H55, IVS23, ey2, or βh1.

Next, we performed ChIP analysis of EKLF binding in 10.5-dpc yolk sac cells isolated from HA/EKLF/TAP mice. The results of this experiment are illustrated in Fig. 1E. As observed in definitive cells, EKLF bound strongly to HS4, HS3, HS2, and H55. Unexpectedly, we discovered that EKLF bound to the promoters of ey2-, βh1-, and βm1-globin genes in these primitive erythroid cells. These results are surprising for several reasons. First, we did not anticipate EKLF binding to the adult β-globin genes in primitive erythroid cells. The adult genes are expressed at very low levels in primitive cells (Fig. 1F), and most models of globin gene switching have predicted that EKLF is not bound to adult globin gene promoters at this stage. However, our ChIP results clearly demonstrate that EKLF binds to the βg-globin gene promoter in primitive cells and that the association is quantitatively equivalent to binding in definitive cells. Secondly, we were surprised to find that EKLF bound efficiently to ey2- and βh1-globin gene promoters in primitive erythroid cells. Although Northern blot and in situ hybridization experiments

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FIGURE 1. Globin gene switching and ChIP analysis of EKLF binding to murine β-globin locus sequences during development. A, reverse transcription-PCR analysis of embryonic/fetal to adult globin gene switching in HA/EKLF/TAP mice. YS is a 10.5-dpc yolk sac; FL is a 14.5-dpc fetal liver; PC is primer control (no RNA). B–F, ChIP analysis of EKLF binding to β-globin locus sequences. Cells were fixed with 1% formaldehyde, and chromatin was prepared for immunoprecipitation. EKLF-bound chromatin was precipitated with anti-HA or anti-GFP beads as a control, and DNA was analyzed by PCR for S′-HS5, -HS4, -HS3, -HS2, -HS1, -ey2, -βH1, and -βT sequences. Sequences between HS2 and HS3 were used as a control (IVS23). 32P-labeled bands were quantitated on a phosphorimaging device, and values for globin locus bands (HS sites and genes) were normalized to MyoD internal control bands. Finally, fold enrichment was calculated by dividing the normalized α-HA/α-GFP values from three different animals. Numbers below the lanes represent the mean fold enrichment ± S.D. B, diagram of the murine β-globin gene locus HbbS haplotype and the position of primer sets used in ChIP experiments. C, ChIP analysis of EKLF binding in Ter119− cells purified from bone marrow. This is a control experiment. D, ChIP analysis of EKLF binding in Ter119+ cells purified from bone marrow. E, ChIP analysis of EKLF binding in a 10.5-dpc yolk sac. F, ChIP analysis of EKLF binding in a 14.5-dpc fetal liver. The results demonstrate EKLF binding to ey2 and βH1 gene promoters in primitive (but not definitive) erythroid cells. BM, bone marrow; M, marker.

have demonstrated that the EKLF gene is expressed in yolk sac erythroid cells (24, 25), previous models of globin gene switching did not predict EKLF binding to ey2- and βH1-globin gene promoters at this developmental stage, because ey2- and βH1-globin genes are expressed at near wild-type levels in EKLF knock-out mice (26, 27). The knock-out result suggested that EKLF is not involved in expression of the embryonic/fetal globin genes. However, the ChIP results presented above clearly demonstrate that EKLF binds to ey2 and βH1 promoters in 10.5-day yolk sac cells and suggest that EKLF plays an important role in globin gene expression at this developmental stage. In EKLF knock-out mice, compensatory CACCC-binding proteins may stimulate ey2 and βH1 expression. However, in wild-type animals, these compensating proteins may not play a major role in globin gene regulation.

There are several possible explanations for the lack of EKLF binding to ey2 and βH1 promoters in definitive cells. One possibility is that repressors bind to these promoters in definitive cells and inhibit EKLF access to the CACCC boxes. Several repressors have been proposed, and mutations of potential repressor binding sites in embryonic/fetal globin gene promoters result in up-regulation of these genes in definitive cells (28–30). However, genetic disruptions of the genes encoding these repressors have not demonstrated long term up-regulation of embryonic/fetal globin genes in definitive erythroid cells, and ChIP experiments have not convincingly demonstrated the binding of these factors to embryonic/fetal globin gene promoters in vivo.

Pattern of Histone H3 Trimethylation at ey2 and βH1 Promoters in Definitive Cells Indicates an Open Chromatin Domain—Another possible explanation for the lack of EKLF binding to ey2 and βH1 CACCC boxes in definitive cells is that a repressive chromatin structure is formed on these genes. To test this possibility, we analyzed the ey2 and βT promoter for histone H3K27 trimethylation, which normally marks “closed” chromatin (31). Two different polyclonal antibodies to trimethylated H3K27 were utilized. Fig. 2 demonstrates that H3K27 trimethylation is not significantly enriched on the ey2 promoter (lanes 4 and 5) compared with the βT promoter (lanes 9 and 10) in 14.5-dpc fetal liver cells. The myoD1 internal control demonstrates that H3K27 trimethylation is highly enriched on this gene, which is repressed in all
FIGURE 2. ChIP analysis of histone H3 trimethylation at the ε2- and β-globin gene promoters in definitive erythroid cells. Chromatin obtained from a 14.5-dpc fetal liver was immunoprecipitated with control rabbit IgG (lanes 1 and 6), anti-trimethyl-histone H3K9 (lanes 2 and 7), anti-trimethyl-histone H3K4 (lanes 3 and 8), and two anti-trimethyl-histone H3K27 antibodies (lanes 4, 5, 9, and 10). Histone H3 trimethylation at the myoD1 gene was analyzed as a control. The results demonstrate that the ε2 promoter contains chromatin marks that are consistent with an open domain in definitive erythroid cells; therefore, the promoter appears to be accessible to EKLF.

FIGURE 3. Western blot analysis of EKLF in primitive and definitive erythroid cells. Ter119<sup>+</sup> cells were isolated from 10.5-dpc yolk sacs and 14.5-dpc fetal livers of the HA/EKLF/TAP homozygous mice. EKLF and GAPDH protein levels in whole cell extracts were determined by Western blot analysis with anti-HA and anti-GAPDH monoclonal antibodies. HA/EKLF/TAP migrated as a doublet of 65 and 70 kDa, and GAPDH (the loading control) migrated as a single band of 34 kDa. The enrichment of HA/EKLF/TAP relative to GAPDH is shown below the gel. The results demonstrate that EKLF levels are increased ~3-fold in definitive compared with primitive erythroid cells.

EKLF Levels in Erythroid Progenitors Change Significantly during Development—A third explanation for the differential binding of EKLF to globin gene promoters in primitive and definitive cells is that the abundance of EKLF changes during development. Changes in the levels of other hematopoietic transcription factors have dramatic effects on gene regulation and lineage specification (37–46), and in many cases, the changes in transcription factor levels result in a change of interacting partners (47). Therefore, we measured EKLF levels in primitive and definitive erythroid cells from homozogous HA/EKLF/TAP mice with anti-HA antibody (Fig. 3). Interestingly, we found that EKLF levels increase ~3-fold in definitive compared with primitive erythroid progenitors (see supplemental Fig. 1 for quantitation). This result is consistent with the ~3-fold increase in EKLF mRNA levels that we previously observed in definitive, compared with primitive, erythroid cells (24). We hypothesize that the ~3-fold increase in EKLF in definitive, compared with primitive, erythroid cells results in a switch of EKLF binding partners and that the new complex binds preferentially to adult β-globin gene promoters. Therefore, adult globin gene family members are preferentially activated in definitive erythroid cells.

EKLF Binding to β-Globin Gene Promoters Does Not Require the LCR—Because EKLF binds to the LCR and to promoters of adult β-globin genes in erythroid cells at all developmental stages, we investigated whether EKLF binding to the β-globin gene promoter requires the LCR. We deleted HS1–HS4 of the LCR (ΔLCR) in murine embryonic stem cells and produced mice that were heterozygous for this 18-kb deletion.ΔLCR animals were bred with HA/EKLF/TAP mice to produce animals that were heterozygous for the ΔLCR allele and homozygous for HA/EKLF/TAP. To distinguish the binding of EKLF at WT and ΔLCR alleles, we used mice containing both HbbS (S) and HbbD (D) haplotypes and performed allele-specific ChIP as described by Sawado et al. (20). HS3 from the HbbS allele is 16 nt longer than HS3 from the HbbD allele. As demonstrated in the control experiments in Fig. 4A, both HS3 alleles were precipitated by anti-HA antibody in wild-type animals (lane 4; one wild-type HbbS allele and one wild-type HbbD allele), and only HS3 from the HbbD allele was enriched in ΔLCR heterozygotes (lane 2; one ΔLCR HbbD allele and one wild-type HbbS allele). When allele-specific ChIP analysis of adult β-globin gene promoters was performed, we found that EKLF was recruited to the adult β<sup>αmol</sup> globin gene promoter of the ΔLCR allele as strongly as to the β<sup>β</sup>-globin gene promoter of the WT allele (Fig. 4B, compare lanes 2 and 4). These results demonstrate that EKLF binding to the adult β-globin gene promoter does not require the LCR. Similar data demonstrating polII, GATA-1, and Fog-1 binding to β-globin gene promoters in the absence of the LCR in vivo suggest that the LCR does not deliver these important proteins to globin gene promoters (14, 20).

However, EKLF is essential for LCR/globin gene interactions (13), and EKLF binding sites in HS3 and HS2 are essential for full LCR activity in primitive and definitive erythroid cells in vivo.<sup>4</sup> Therefore, EKLF binding to both LCR and globin gene promoter sequences in the yolk sac, fetal liver, and bone marrow appears to be critical for correct globin gene regulation during development.

DISCUSSION

The “Results” presented above clearly demonstrate that EKLF binds to ε2 and β<sub>h1</sub> promoters in primitive erythroid cells. The most straightforward interpretation of these data is that EKLF activates ε2 and β<sub>h1</sub> gene expression at this developmental stage. EKLF has two powerful transcription activation domains (25, 48), and the binding of EKLF to the ε2 and β<sub>h1</sub> promoters strongly suggests a functional role for this important transcription factor in primitive erythroid cells. Although ε2- and β<sub>h1</sub>-globin genes are expressed at relatively high levels in EKLF knock-out mice, compensatory CACCC box-binding proteins may be responsible for this effect. Interestingly, yeast one-
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![Diagram](Image)

**FIGURE 4.** ChIP analysis of EKLF binding to β-globin promoter in the absence of the LCR. Ter119+ erythroid cells were purified from the bone marrow of either ΔLCR (A and B, lanes 1 and 2) or wild-type (A and B, lanes 3 and 4) mice. Chromatin DNA was prepared and precipitated with anti-HA or -GFP antibodies and analyzed by PCR. A, control experiment. Shown is allele-specific analysis of EKLF binding to HS3 in ΔLCR (D)/WT (S) mice by ChIP. As expected, only HS3 from the wild-type HbbS allele was enriched by anti-HA antibody by ΔLCR (D)/WT (S) mice (compare lane 2 with lane 3). B, allele-specific analysis of EKLF binding to the promoter-proximal region of the βmaj- and βmin-globin genes in ΔLCR (D)/WT (S) mice. The results demonstrate that EKLF binds strongly to the βmaj gene promoter in the absence of the LCR (compare lanes 2 and 4).

EKLF binding to the promoter-proximal region of the LCR. As expected, only HS3 from the wild-type HbbS allele was enriched by anti-HA antibody in ΔLCR (D)/WT (S) mice and precipitated with anti-HA or -GFP antibodies and analyzed by PCR.

**FIGURE 5.** Model for globin gene switching during development. A 3-fold increase of EKLF (red ovals) in definitive, compared with primitive, erythroid cells results in a switch of EKLF partners (yellow and blue ovals). The complex formed in primitive cells binds equivalently to embryonic/fetal and adult globin gene CACCC boxes, but ε2 and βh1 genes are expressed preferentially due to LCR proximity. The EKLF complex formed in definitive cells binds to adult (but not to embryonic/fetal) globin gene CACCC boxes and stimulates interactions with the LCR; therefore, the adult genes are expressed preferentially at this developmental stage. Purification of EKLF complexes from yolk sac and fetal liver should identify EKLF binding partners. In this model, primitive and definitive stage-specific transcription factors are not required; temporal-specific changes in factor abundance are sufficient to mediate a switch in globin gene expression.

hybrid screens of yolk sac cDNA libraries identify predominately EKLF, but also Sp1 and Sp3, as embryonic/fetal globin gene CACCC box-binding proteins. Therefore, Sp1 and Sp3 may bind to embryonic/fetal globin gene CACCC boxes in the absence of EKLF and stimulate ε2-, βh1-, or γ-globin gene expression.

A simple model for globin gene switching that is consistent with the EKLF binding pattern described above is illustrated in Fig. 5. EKLF forms different complexes in primitive and definitive erythroid cells, and these different complexes direct globin gene switching. In primitive cells, the EKLF complex (Fig. 5, red and yellow ovals) binds to ε2-, βh1-, and adult β-globin gene CACCC boxes equivalently; however, ε2 and βh1 genes are preferentially expressed, because these genes are more proximal to the LCR. An alternative explanation for the preferential expression of ε2- and βh1-globin genes in primitive cells is that an EKLF repressor complex binds to the adult globin gene promoters at this stage (49, 50). In definitive cells, a new EKLF complex (Fig. 5, red and blue ovals) is formed as a result of the 3-fold increase in EKLF protein levels. This complex binds to adult, but not to embryonic/fetal, globin gene CACCC boxes; therefore, the adult genes are expressed preferentially at this developmental stage. This model does not exclude an important role for other transcription factors, such as KLF2 (51), in globin gene switching but emphasizes the essential role of EKLF. Also, other cis-acting elements may be necessary for correct globin gene switching. However, mutation of the γ-globin gene CACCC box is sufficient to produce a major change in developmental specificity (that is, high level expression of adult β-globin genes in primitive erythroid cells (9, 10)); therefore, differential binding of EKLF to embryonic/fetal and adult globin gene CACCC boxes appears to be critical for correctly regulated globin gene switching.

Previously published functional data are consistent with this model. Underexpression of EKLF in heterozygous EKLF knock-out mice results in delayed switching (15, 16), and overexpression of EKLF in transgenic mice results in premature globin gene switching (17). We favor a simple model for globin gene switching in which temporal-specific changes of EKLF abundance result in 1) a switch of EKLF partners, 2) differential binding of EKLF complexes to globin gene promoters, and 3) developmental stage-specific globin gene expression. Purification of EKLF complexes from primitive and definitive erythroid progenitors should identify EKLF binding partners. In this model, primitive and definitive stage-specific interactions of globin gene family members with the powerful locus control region.

Acknowledgments—We thank Jackie McLeroy and Clark Kelley for outstanding animal care.

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