Activation of δ-globin gene expression by erythroid Krupple-like factor: A potential approach for gene therapy of sickle cell disease

David Donze  
Department of Biochemistry and Molecular Genetics

Paxson H. Jeancake  
Department of Biochemistry and Molecular Genetics

Tim M. Townes  
Department of Biochemistry and Molecular Genetics

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Activation of \( \delta \)-Globin Gene Expression by Erythroid Krupple-Like Factor: A Potential Approach for Gene Therapy of Sickle Cell Disease

By David Donze, Paxson H. Jeancake, and Tim M. Townes

**CURRENT GENE THERAPY strategies** for sickle cell disease focus on transduction of hematopoietic stem cells with viral vectors containing antisickling globin genes.\(^\text{14}\) Although this is a viable approach, expression of therapeutic levels of \( \beta \)-globin mRNA and protein from transduced genes may be difficult to achieve (Palmer et al.,\(^\text{5}\) Weatherall,\(^\text{6}\) McCune et al.,\(^\text{7}\) and M. Sadelain, personal communication, June 1996). An alternative approach is to transduce stem cells with a transcription factor gene encoding a protein that specifically enhances endogenous \( \delta \)-globin gene expression. In this case, relatively low levels of a novel transcription factor may stimulate high levels of \( \delta \)-globin gene expression.

Hemoglobin A2 (HbA2; \( \alpha_2\beta_2 \)) is a powerful inhibitor of HbS (\( \alpha_2\beta_2\)) polymerization;\(^\text{8}\) however, HbA2 levels are lowest in sickle cell patients and in normal individuals (2% to 3% of total Hb).\(^\text{9}\) One reason for low \( \delta \)-globin expression is the defective CACCC box at -50 in the \( \delta \)-globin gene promoter (see below); this region is known to be important for \( \delta \)-globin gene expression.\(^\text{10-12}\) The major CACCC box-binding protein in the adult \( \beta \)-globin gene is the erythroid-specific, zinc finger transcription factor EKLF (Erythroid Krupple-like factor).\(^\text{13}\) This protein binds to the \( \delta \)-globin CACCC box and, in conjunction with proteins bound to locus control region (LCR) sequences, activates high-level \( \beta \)-globin gene expression.\(^\text{14-16}\) Our goal is to design a modified EKLF that binds specifically to the defective \( \delta \)-globin gene promoter and enhances \( \delta \)-globin gene expression. The results described below show the potential of this approach and suggest that modified EKLF proteins could be used in gene therapy strategies to enhance HbA2 synthesis and inhibit erythrocyte sickling.

**MATERIALS AND METHODS**

**Plasmid constructions.** The construction of plasmid HS2 \( \beta \)-Luciferase has been described.\(^\text{14}\) The plasmid contains the 1.5-kb Kpn I-\( \beta \)Globin II human LCR HS2 fragment upstream of the -265 to +48 region of the \( \beta \)-promoter with Bgl II ends attached to the primers. The template used was a human globin locus subclone containing the entire \( \beta \)-gene, and the primer sequences (\( \beta \)Globin II sites underlined) were as follows: upstream, 5'-CTCGAGCTACGAGATGCCTGAAATTG-GATACTG-3'; and downstream, 5'-CTCGAGCTACGAGATGCCTGAAATTG-GATACTG-3'. The PCR products were digested with Bgl II and cloned into the plasmid HS2[\( \beta \)],\(^\text{17}\) Modified \( \delta \)-promoters were made by the megaprimer mutagenesis method.\(^\text{18-20}\) Using the same outside oligos listed above with the following mutagenic oligos: \( \delta \)-globin, 5'-TTTCTATTCTAAGACAAATAC-CAACACCATGTTTATCTAACCACCAATTGC-3'; \( \delta \)-globin, 5'-TTTCTATTCTAAGACAAATAC-CAACACCATGTTTATCTAACCACCAATTGC-3'; this consensus EKLF-binding sites are in bold, and these fragments were cloned into HS2[\( \beta \)] as above.

To construct the HS2 \( \beta \)-globin plasmids, the 3.0-kb Xba I-Sph I fragment of the \( \delta \)-globin gene from -400 to about 1 kb downstream of the poly A site was first subcloned into \( \mu \)C19. The modified \( \delta \)-promoters (amplified from the -400 Xba I site to the Sal I site at -13) were then synthesized by megaprimer mutagenesis using the same mutagenic oligos listed above and the outside primers 5'-AGTTTAAGACTGACGAT-3' (starting at -460 of the \( \beta \)-promoter) and 5'-CTTCTCTACGAGATCTAG-3' (just downstream of the translation start). These PCR fragments were cut with Xba I and Sal I and were used to replace the corresponding wild-type fragment in the \( \delta \)-gene \( \mu \)C19 subclone. The \( \delta \)-globin promoter was constructed in the same fashion, using the mutagenic oligos 5'-GAAGGTTCAGTTTCTTTCACTGACGAT-3' and 5'-GAAGGTTCAGTTTCTTTCACTGACGAT-3'. Each of these \( \delta \)-genes was cut out of the \( \mu \)C19 vector with Xba I and HindIII, blunted with S1 nuclease, and cloned into plasmid DNA. The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.\(^\text{© 1996 by The American Society of Hematology.}\)

From the Department of Biochemistry and Molecular Genetics, Schools of Medicine and Dentistry, University of Alabama at Birmingham, Birmingham, AL.

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Address reprint requests to Tim M. Townes, PhD, Department of Biochemistry and Molecular Genetics, University of Alabama at Birmingham, Schools of Medicine and Dentistry, 845 19th St S, Birmingham, AL 35294.

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transcriptional start site as is the p-globin promoter EKLF-binding site. The SCAc' promoter contains the EKLF-binding site at bases -85 to -93; this is the same distance from the transcriptional start site as is the β-globin promoter EKLF-binding site.

Fig 1. Comparison of the human β- and δ-promoters and modification of the δ-promoter to contain EKLF-binding sites. (A) The β-globin locus on human chromosome 11. (B) The β- and δ-globin promoters were aligned using the GAP program of the GCG/Wisconsin package; only the regions of interest are shown. The consensual EKLF-binding site in the β-globin promoter (CCACACCCCT) is located at bases -85 to -93 from the transcription start site, and this sequence is underlined. The wild-type δ-globin promoter region has a partial EKLF-binding site (ACCCCT) at bases -81 to -85. To create the modified δACc' promoter, site-directed mutagenesis was used to convert the partial site to a consensual EKLF-binding site. The δACc' promoter contains the EKLF-binding site at bases -85 to -93; this is the same distance from the transcriptional start site as is the β-globin promoter EKLF-binding site.

5'HS2(K-P)/β,22 which was cut with Cla I and S1-blunted. All PCR-amplified regions in each of these constructs were completely verified by dideoxy sequencing using the Sequenase kit (US Biochemicals Corp, Cleveland, OH).

The pCIneo-GAL4,1.147,EKLF plasmid was constructed in two steps: A 0.8-kb Neo I-Mspl I cDNA fragment containing the coding sequence (amino acids 2-275) of the murine EKLF activation domain (from plasmid pSG5-EKLF)13 was blunt-ended with S1 nuclease and cloned in-frame into the GAL4 DNA-binding domain expression plasmid pBXG1 (gift from M. Ptashne, Harvard University, Cambridge, MA). The pBXG1/EKLF plasmid was cut with HindIII and BamHI, and the 1.3-kb GAL4,1.147,EKLF fragment was isolated, S1-nuclease-blunted, and cloned into pCIneo (Promega Corp) cut with Sma I. As a control, the 0.5-kb GAL4-binding domain HindIII-BamHI fragment from pBXG1 was cloned into pCIneo as above [pCIneo-GAL4,1.147].

Murine erythroleukemia (MEL) cell transfections. MEL cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS). For transient transfections, cells were washed once and resuspended to 10⁶ cells/mL in RPMI-1640 media without serum. For transient luciferase assays, 40 µg of HS2 promoter/Luciferase plasmids were mixed with 5 µg internal control plasmid CMV-β gal (Clontech Lab, Palo Alto, CA) in 100 µL phosphate-buffered saline and were placed in a 0.4-cm gap electroporation cuvette (Bio-Rad, Hercules, CA). MEL cells (5 x 10⁶) in 0.5 mL RPMI-1640 media (without serum) were added to each cuvette, and cells were electroporated at 260 V and 960 µF in a Bio-Rad Gene Pulser. The contents of each cuvette were transferred into a 100-mm plate containing 10 mL DMEM/10% FBS and were incubated for 8 hours. After 8 hours, dimethyl sulfoxide (DMSO) was added to 1.65% to induce erythroid differentiation. This 8-hour preincubation before DMSO induction increased cell viability and gene expression levels severalfold when compared with those of cells induced immediately after electroporation. Cultures were incubated for 3 days, and then cell extracts were assayed for luciferase and β-galactosidase as described.7,14 Transient transfections were performed 3 times in triplicate.

Transfection of MEL cells to produce G418-resistant populations was performed essentially as described by Collins et al.25 A total of 20 µg of HS2 δ-δ plasmids (with δ, δACc', or δACc' promoters) was mixed with a 1:10 molar ratio of plasmid pgk-neo (the murine phosphoglycerate kinase promoter driving neomycin phosphotransferase; a gift from R. Mortensen, Harvard University). Both plasmids were linearized with Kpn I, precipitated, and dissolved in 100 µL phosphate-buffered saline. The plasmid mix was placed into a 0.4-cm electroporation cuvette, and 2 x 10⁶ MEL cells (prepared as above) in 0.5 mL RPMI-1640 media were added. Cells were electroporated at 200 V and 960 µF and were transferred to 30 mL DMEM/10% FBS in a 75-cm² culture flask. After 48 hours, G418 was added to a final concentration of 300 µg/mL, and resistant cell populations were selected for 2 weeks. Cells were then induced with 1.65% DMSO for 3 days, and total RNA was extracted as described.24 The HS2 δACc'-β-transcripts were performed as above, using a 1:10 molar ratio of the pCIneo plasmids, which were linearized with Xmn I.

Analysis of relative levels of δ- and β-transcripts. The relative levels of δ- to β-globin transcripts were determined by the single nucleotide primer extension (SNuPE) assay, which is based on detecting single known nucleotide differences in allelic RNAs.25 Briefly, RNA from MEL cells transfected with HS2 δ-δ constructs was amplified by reverse transcription-PCR (cDNA cycle kit; Invitrogen, San Diego, CA) using oligo dT to prime the cDNA reaction. The PCR primers were as follows: upstream, 5'-TGTTCA-CATGCAACCTCAAAC-3'; and downstream, 5'-TGAAGTTTCT-CAGGATCACC-3'. There are no differences in δ- and β-globin sequences in these regions, and the primers do not amplify mouse...
ACTIVATION OF δ-GLOBIN EXPRESSION BY EKLF

A.

HS2

β, δ, or δCAC

promoter

Luciferase

Kpn I

Bgl II

-265

+48

B.

Relative Luciferase Activity,

as % of HS2-δ/Luciferase

Promoter:

β

δ

δCAC-1

δCAC-3

Fig 2. Transient expression of HS2 δ-promoter/Luciferase plasmids in MEL cells. (A) Schematic diagram of δ-globin promoter/reporter plasmids. The 1.5-kb Kpn I-Bgl II LCR HS2 fragment was linked to the -265 to +48 wild-type β, δ, and modified δ-globin promoters described in Fig 1. These promoters were used to drive luciferase reporter gene expression in transiently transfected MEL cells. Luciferase activity was normalized to β-galactosidase expression from an internal control CMV-β-galactosidase plasmid. (B) HS2 β/Luc expression was normalized to 100%. Inclusion of the consensus EKLF site into the δCAC-1 and δCAC-2 promoters increased HS2 δ/Luc expression from 10% to 56% and 99% of HS2 β/Luc, respectively.

globin mRNAs (data not shown). These oligos specifically amplify the corresponding 341-bp fragments of both the human δ- and β-globin cDNAs, and the fragments were purified by agarose gel electrophoresis. The detection oligo, 5'-CTTCTCTCAGGAGTGATCAC-3', hybridizes to the identical sense strand sequence of both δ- and β-globin cDNA PCR products (corresponding to nucleotides +77 to +60 of each message) and is extended to base +59 by α32P-deoxyadenosine triphosphate in the δ reaction or by α32P-deoxyguanosine triphosphate for the β reaction. Labeled products were resolved on a 15% polyacrylamide/8 M urea gel, and bands were quantitated on a Molecular Dynamics Model 425 Phosphorimager (Molecular Dynamics, Sunnyvale, CA). Linearity of the assay was verified as described by mixing experiments using RNA from MEL cells transfected with either HS2 β or HS2 β-promoter/δ-gene as sources containing the individual messages (data not shown).

RESULTS

The β- and β-globin genes are located at the 3' end of the β-globin locus on human chromosome 11 (Fig 1A). Comparison of the β- and β-globin proximal promoters (Fig 1B) shows two key differences: the δ-globin promoter lacks consensus CACCC and CAAAT boxes, which are important for adult β-globin gene expression, EKLF, which binds to the β-globin CACCC box (CCACCCCT) from bases -93 to -85 of the promoter, is critical for β-globin gene expression; however, EKLF does not bind to this region of the δ-globin promoter (data not shown). To test the feasibility of using a modified EKLF to activate δ-globin gene expression, we determined whether a δ-globin promoter containing a β-globin EKLF-binding site would increase expression by recruiting endogenous cellular EKLF in transient transfection assays. We replaced the defective EKLF-binding site in the δ-globin promoter (TGAAACCCCT from -89 to -81; see Fig 1) with the consensus CCACCCCT to construct the plasmid HS2 δCAC-1/Luciferase. We also constructed a modified δ-globin promoter that contained the CCACCCCT sequence at -93 to -85, so that the EKLF site would be positioned the same distance from the transcription start site as it is in the β-globin promoter (HS2 δCAC-2/Luciferase).

Figure 2 shows the relative expression levels of these modified δ-promoter/Luciferase reporter constructs when linked to an LCR HS2 fragment and transiently transfected into MEL cells. In this assay, HS2 δ/Luc was expressed at 10% of the level of HS2 β/Luc. When the consensus EKLF site was placed in the δ-globin promoter at the -81 to -89 site (HS2 δCAC-1/Luc), expression was increased approximately fivefold, to 56% of HS2 β/Luc expression. HS2 δCAC-2/Luc, with the consensus EKLF site at -85 to -93, expressed the reporter gene at 99% of HS2 β/Luc. These results show that the major defect of the δ-globin gene proximal promoter is the lack of a consensus EKLF-binding site.

In addition to promoter defects, low δ-globin expression has been attributed to other differences between the δ- and β-globin genes. The δ-gene lacks intragenic enhancers that augment β-globin expression, and δ-globin mRNA is less stable than β-globin mRNA. To assess the effects of these differences on δ-ac and δ-ac expression, we constructed complete δ-globin genes (see Materials and Methods) containing the δ, δ-ac, and δ-ac promoters (Fig 3A). These δ-globin genes were used to produce HS2 δ-β constructs. The plasmids were then cotransfected into MEL cells with a pgk-neo selectable marker, and G418-resistant populations were selected. After differentiation was induced with DMSO, RNA was extracted and the relative levels of δ- and β-globin mRNAs were determined by the SNuPE assay.5 Table 1 shows the results of these experiments. The level of δ-globin mRNA (δβ + δ) in MEL cells was 3.0% ± 1.3% for HS2 δ-β, 14.9% ± 7.4% for HS2 δ-ac-1-β, and 22.0% ± 9.0% for HS2 δ-ac-2-β. These results show that the insertion of a consensus EKLF-binding site in the δ-globin promoter significantly enhances δ-globin gene expression in a construct that contains the entire δ-globin gene. A representative example of the SNuPE assay is shown in Fig 3B; δβ + δ-globin mRNA in this experiment was 3.6% for HS2 δ-β, 24.7% for HS2 δ-ac-1-β, and 34.8% for HS2 δ-ac-2-β. As a control, δ- and β-globin mRNA levels were determined for human reticulocyte RNA (Fig 3B); the δβ + β level was 0.6% ± 0.2%.

These results suggest that EKLF binding to the δ-globin gene promoter significantly stimulates expression; however, the experiments do not exclude the possibility that other CACCC-binding factors are responsible for this increase. To confirm that the EKLF activation domain can enhance δ-globin gene expression, we constructed an HS2 δGAL4-β plasmid in which the consensus GAL4-binding site replaced sequences from -80 to -99 of the δ-globin gene promoter...
Expression of modified δ-globin genes in MEL cells stably transfected with HS2 δ-β constructs. (A) Schematic diagram of HS2 δ-β construct. The parental HS2 δ-β construct contained a 1.9-kb Kpn I-Pvu II HS2 fragment, a 3.0-kb δ-globin gene beginning at base -400 from the transcription start site, and a 4.1-kb β-globin gene beginning at base -815 from the transcription start site. The corresponding δCAC-1 and δCAC-2 modifications were inserted to create HS2 δCAC-1-β and HS2 δCAC-2-β. These constructs were transfected into MEL cells with a pgk-neo-selectable marker, and G418-resistant populations were selected. The cells were induced to differentiate with DMSO, and RNA was extracted for analysis. (B) The relative levels of δ- and β-globin mRNAs were determined by the SNuPE assay and quantitated by phosphorimager analysis. Inclusion of the consensus EKLF site into the δCAC-1- and δCAC-2-globin genes increased δ-globin mRNA levels from 3.6% to 24.7% and 34.8% of total δ + β mRNA, respectively.

Table 1. Relative Levels of δ-Globin Transcripts From MEL Transfections and Human Reticulocyte Samples as Percentages of Total Human δ + β Transcripts

<table>
<thead>
<tr>
<th>Experiment no.</th>
<th>HS2 δ-β</th>
<th>HS2 δCAC-1-β</th>
<th>HS2 δCAC-2-β</th>
<th>Reticulocyte</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.6</td>
<td>24.7</td>
<td>34.8</td>
<td>0.5</td>
</tr>
<tr>
<td>2</td>
<td>1.9</td>
<td>10.0</td>
<td>15.0</td>
<td>0.7</td>
</tr>
<tr>
<td>3</td>
<td>1.6</td>
<td>5.8</td>
<td>12.3</td>
<td>0.8</td>
</tr>
<tr>
<td>4</td>
<td>4.7</td>
<td>19.0</td>
<td>25.7</td>
<td>0.3</td>
</tr>
<tr>
<td>Average</td>
<td>3.0 ± 1.3</td>
<td>14.9 ± 7.4</td>
<td>22.0 ± 9.0</td>
<td>0.6 ± 0.2</td>
</tr>
</tbody>
</table>

Expression of modified δ-globin genes in MEL cells stably transfected with HS2 δ-β constructs. These constructs were transfected into MEL cells with a pgk-neo-selectable marker, and G418-resistant populations were selected. The cells were induced to differentiate with DMSO, and RNA was extracted for analysis. The relative levels of δ- and β-globin mRNAs were determined by the SNuPE assay and quantitated by phosphorimager analysis.

DISCUSSION

Current gene therapy approaches for treatment of sickle cell disease involve viral transduction of hematopoietic stem
Fig 4. GAL4/EKLF activates δ-globin gene expression in an HS2 δGAL4-β construct. (A) Schematic drawing of the reporter plasmid HS2 δGAL4-β. This construct contains a consensus GAL4-binding site (expanded) replacing δ-globin promoter bases -80 to -99. (B) PCIeo effector constructs expressing either the GAL4 DNA-binding domain (amino acids 1-147) alone or GAL4-δGAL4 fused to the EKLF activation domain (amino acids 2-275). (C) HS2 δGAL4-β was stably transfected into MEL cells with either PCIeo-GAL4-D147, or PCIeo-GAL4-δGAL4/EKLF, and relative transcript levels were determined by the S NuPE assay. The level of human δ/β was 4.4% in cells containing HS2 δGAL4-β, 13.3% in cells containing HS2 δGAL4-β plus PCIeo-GAL4-D147, and 36.6% in cells containing HS2 δGAL4-β plus PCIeo-GAL4-δGAL4/EKLF. These results show that a modified EKLF can significantly increase δ-globin gene expression.
Table 2. Relative Levels of $\delta$-Globin Transcripts From MEL Cells Transfected With HS2-$\delta$GAL4-$\beta$ and GAL4-11.1d71 Expression Constructs as Percentages of Total $\delta + \beta$ Transcripts

<table>
<thead>
<tr>
<th>Experiment no.</th>
<th>HS2 $\delta$GAL4-$\beta$ + GAL4-11.1d71</th>
<th>HS2 $\delta$GAL4-$\beta$ + GAL4-11.1d71/EKLF</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>13.3</td>
<td>36.6</td>
</tr>
<tr>
<td>2</td>
<td>8.4</td>
<td>30.2</td>
</tr>
<tr>
<td>3</td>
<td>6.7</td>
<td>16.8</td>
</tr>
<tr>
<td>4</td>
<td>11.3</td>
<td>27.3</td>
</tr>
<tr>
<td>Average</td>
<td>$9.9 \pm 2.5$</td>
<td>$27.8 \pm 7.1$</td>
</tr>
</tbody>
</table>

GAL4/EKLF activates $\delta$-globin gene expression in an HS2 $\delta$GAL4-$\beta$ construct. HS2 $\delta$GAL4-$\beta$ was stably transfected into MEL cells with either pClneo-GAL4, or pClneo-GAL4/11.1d71/EKLF, and relative transcript levels were determined by the SNuPE assay. These results show that a modified EKLF can significantly increase $\delta$-globin gene expression.

cells with antisickling globin genes. Although this is a viable approach, expression of therapeutic levels of $\beta$-globin mRNA and protein from transduced genes may be difficult to achieve (Palmer et al., Weatherall, McCune et al., and M. Sadelain, personal communication, June 1996). An alternative approach is to transduce stem cells with a transcription factor gene that specifically enhances endogenous $\delta$-globin gene expression. In this case, relatively low levels of a novel transcription factor may stimulate high levels of $\delta$-globin gene expression. We propose that modified EKLFs, which contain zinc fingers designed to bind specifically to the defective $\delta$-globin CACCC box, may be used in genetic therapy approaches to increase HbA2 levels and to inhibit erythrocyte sickling. The experiments in this report were performed to test the feasibility of this approach.

The results presented here show that the major defect in the proximal $\delta$-promoter is the lack of a consensus EKLF-binding site. Insertion of an EKLF-binding site at $-85$ to $-93$ increased promoter activity to levels equivalent to those of $\beta$. Although other defects including the lack of intragenic enhancers and reduced $\delta$-globin mRNA stability inhibit $\delta$-globin gene expression, correction of the CACCC defect resulted in $\delta$ expression levels as high as 34.8% of total $\delta + \beta$ mRNA in constructs that contained complete $\beta$- and $\beta$-globin genes. The results of the GAL4/EKLF experiments confirm that a modified EKLF can activate $\delta$-globin gene expression to 36.6% of total $\delta + \beta$. HbA2 ($\alpha_2\beta_2$) is a powerful inhibitor of HbS ($\alpha_2\beta_2^S$) polymerization; in vitro studies show that HbA2 is as effective as HbF ($\alpha_2\gamma_2$) at inhibiting the polymerization that causes erythrocyte sickling. Platt et al. recently showed that HbF levels greater than 8.6% significantly enhance the survival of patients with sickle cell disease. Elevation of HbA2 levels from the normal range of 2% to 3% to levels that are greater than 8.6% should similarly enhance survival.

How can EKLF be modified to bind to the defective $\delta$-globin gene promoter and enhance expression? Recently, several groups have used the phage display system to select modified zinc-finger proteins that bind to novel sites. In an elegant set of experiments, Choo et al. showed that a modified Zif268 transcription factor, which was selected by phage display, could bind to the BCR-ABL translocation break point and inhibit BCR-ABL oncogene expression. The results show that zinc finger proteins can be functionally modified to exert a specific physiological effect. We are presently using phage display to select EKLF variants that bind to wild-type $\delta$-globin promoter sequences with high affinity and specificity. These “$\delta$-EKLF” proteins will then be tested in MEL cells containing the HS2 $\delta$-$\beta$ construct to determine the level of $\delta$-globin gene activation. The results described above suggest that $\delta$-globin levels greater than 8.6% of total $\delta + \beta$ mRNA may be possible. The model depicted in Fig 5 shows this effect. The efficacy of $\delta$-EKLF...
expression will be tested in transgenic animals containing the human $\delta$- and $\beta$-globin genes and in hematopoietic stem cells obtained from sickle cell patients.

**ACKNOWLEDGMENT**

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