The β Globin 3' Enhancer Element Confers Regulated Expression on the Human γ Globin Gene in the Human Embryonic-Fetal Erythroleukemia Cell Line K562

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We have constructed fusion genes comprised of γ and β globin elements and globin sequences linked to neomycin resistance (neo) genes to define the cis acting sequences responsible for developmental stage-specific expression and induction of fetal globin genes in embryonic-fetal erythroleukemia K562 cells. The results indicate that the γ promoter is required for proper initiation of transcription. However, the accumulation of γ globin transcripts in response to hemin induction requires the additional presence of either γ intervening sequence 2 or the 3' enhancer element of the β globin gene. Thus, the γ promoter may provide the elements for developmental stage-specific gene expression during fetal life. By contrast, the β 3' enhancer is erythroid-specific but not developmental stage- or gene-specific.

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K562 CELLS ARE A HUMAN erythroleukemia cell line that expresses the endogenous ε and γ genes but not the endogenous β globin genes. In addition, there is an accumulation of ε and γ globin gene transcripts when these cells are grown in hemin, a compound that induces erythroid maturation. Transfected ε genes are expressed, whereas transfected β genes are not expressed. The lack of endogenous β globin gene expression occurs despite the fact that K562 cells contain a functional β gene when genomic clones containing K562 β genes are expressed in a transient expression system. In addition, when globin gene transcription is analyzed in transient heterokaryons generated from the fusion of murine erythroleukemia cells (MELC) with K562 cells, activation of the human β globin gene as well as the mouse embryonic globin gene is observed. Taken together, these results suggest that the differential globin gene expression observed in K562 cells results from the interaction of embryonic and fetal factors with fetal globin gene sequences, and the lack of β gene expression is caused by either the absence of factors required to activate β gene transcription or the presence of negative regulatory factors for β genes in these cells. Thus, K562 cells represent a system to identify the cis-acting sequences and the trans-acting factors that interact with these sequences to activate γ globin gene expression in a fetal erythroid environment.

Previous analysis of fusion globin gene transcription indicates that γ 5' promoter sequences are sufficient for correct initiation of transcription, and suggests that γ intervening sequence 2 (IVS 2) may play a role in the induction of globin transcripts in response to hemin stimulation. Several studies have evaluated the regions within and surrounding the β globin gene required for regulated expression in MELC and transgenic mice. These analyses have positioned an important regulatory element for β globin gene transcription within a Pst I/Pst I fragment of 250 basepairs (bp) located 550 to 800 bp 3' to the polyadenylation site of the β globin gene. This region has been designated as the β 3' enhancer sequence. The expression of globin genes in transgenic mice suggests that the β 3' enhancer as well as an additional element located within exon 3 of the β globin gene are essential for erythroid-specific expression and developmental stage-specific expression.

The analysis of fusion gene transcription and induction in K562 cells provides an important approach to identify the functional significance of regulatory sequences on human γ globin gene expression in vivo. We have now constructed both γβ and globin-neo fusion genes to evaluate the roles of γ promoter, γ IVS 2, β 3' enhancer, and γ intragenic sequences on expression and induction in embryonic-fetal erythroid cells. Our results indicate that either γ IVS 2 or the β 3' enhancer is required for induction of γ globin gene expression in K562 cells.

MATERIALS AND METHODS

Fusion gene constructs. The fusion gene constructs are shown in Fig 1. The top line shows a schematic representation of the β globin gene and the important regulatory sequences are indicated. The γβ fusion genes were constructed in SP6, a pBR 322 derivative, by substituting regions from each of these genes using restriction endonuclease sites that generate compatible ends. The fusion genes were then subcloned into pSV2neo (Fig 1). γ promoter (γβ) contains the γ 5' EcoRI-NcoI fragment of 1.3 kilobases (kb) fused to the β globin gene Neo 1-Bgl II fragment of 3.4 kb that includes the β 3' enhancer. This 3' enhancer element was deleted in γ promoter βΔ 3' (γβΔ3') by removing an Acc 1-EcoRV fragment of 1.3 kb from the β 3' flanking region. γ promoter by IVS 2 Δ 3' (γβγΔ3') was generated by substituting the BamHI-EcoRI fragment containing IVS 2 and the identical BamHI-EcoRI fragment containing IVS 2 in γβΔ3'.

The γ promoter neo fusion gene (γneo) was made by substituting the 1.3-kb EcoRI-Acy I fragment extending from the 5' region of the γ gene into the 5' untranslated region of the SV40 promoter region in pSV2neo. To do this, pSV2neo was digested with Nde I, the cohesive ends were filled in with the large fragment of DNA polymerase I (Klenow fragment), and EcoRI linkers were added. The modified pSV2neo was digested with Bgl II and the cohesive ends were filled with Klenow to generate a Cla I site. The pSV2neo

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RESULTS

The transcription of pools of clones or individual clones transfected with γβ fusion genes was evaluated by RNAase protection analysis using a fusion probe complementary to the 5' end of the transcript (Fig 2). The results of these analyses are interchangeable. When individual clones are induced, pools of these clones are also induced. Pools are presented to provide a representation of more individual clones than can be depicted by the results of individual clones alone. γβ is both transcribed and induced in pools of clones (Fig 2A), and with over 50% of individual clones (data not shown). When the β 3' enhancer is deleted (γβΔ3'), the accumulation of transcripts in pools of clones transfected decreases in response to hemin stimulation (Fig 2B). This result suggests that the β 3' enhancer contained on the construct in Fig 2A is required to confer inducible expression on the γβ fusion gene.

Figure 2C shows the transcription of γβγΔ2Δ3'. The only difference between this construct and γβΔ3' is that γ IVS 2 has been substituted for β IVS 2. When this substitution is made, this gene is now inducible in 50% of the lines, indicating that γ IVS 2 has a similar effect to that of the 3' β enhancer in increasing induction.

We have rarely observed a frequency of induction greater than 50% with any genes that we have transfected into K562 cells or MELC

"induction of this fusion gene." By contrast, the γneoR fusion gene was more inducible (γneoR) than γneoR alone (Fig 3). Additional γneoR clones without an added β 3 enhancer were analyzed in these experiments, and, as previously, showed no induction (data not shown). These data indicate that the β 3 enhancer element is capable of conferring induced expres-
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Fig 2. RNAase protection analysis of transcription of γβ globin fusion genes. RNA was prepared from pools of clones (A and B) or individual clones (C) grown in the absence (−) or presence (+) of hemin. The 5' fusion probe is indicated at the bottom and the protected fragment of 146 nucleotides is indicated by the arrows at the right. For all the panels, M, marker; p, probe; C, control K562 RNA; p1, p2, etc, RNA from pools of clones; 1, 2, etc, RNA from individual clones; −, cells uninduced; +, cells induced with 20 μmol/L hemin. (A) RNA from pools of clones transfected with γβ containing the 3' β enhancer (Fig 1, 1). (B) RNA from pools of clones transfected with γβA3' (Fig 1, 2). (C) The RNA from individual clones transfected with γβγ2A3' containing the substitution of γ IVS 2 (Fig 1, 3).

sion on a heterologous gene containing only the γ promoter region, and lacking γ IVS 2 and γ coding sequences.

The amount of RNA analyzed in all the experiments was assessed by agarose-formaldehyde gel electrophoresis, and it was shown that similar amounts of RNA were present in samples before and after hemin induction (data not shown). Neo expression was analyzed in some experiments and no differences were found in the signal before and after induction (data not shown). Actin expression was used as an additional internal control in the experiments shown in Fig 3, and again shows that similar amounts of RNA are present in samples from induced and uninduced cells. There was an excellent correlation between the relative amounts of RNA present in induced and uninduced cell RNA samples when the acrylamide gel RNA data and the actin data are compared. In addition, as we have shown previously, a threefold to fivefold increase in endogenous γ globin gene expression was shown in all samples by visualization of a 48-bp fragment representing exon 3 of the γ globin gene with the γβ-neo fusion probes used (data not shown).

DISCUSSION

We have analyzed the expression of γ and β globin fusion genes to evaluate the role of the γ promoter, γ IVS 2, and β 3' enhancer sequences on the expression and induction of γ globin genes in erythroid cells. An effect of the substitution of β or δ IVS 2 for β IVS 2 in fusion globin genes has been previously reported. When γ IVS 2 is substituted for β IVS 2 in a β globin gene, induction of this fusion gene following transfection into K562 cells is observed with a 3' β probe. However, the transcripts are not appropriately initiated because the β globin promoter does not seem to function in these embryonic-fetal cells. When the converse fusion gene is made and β IVS 2 is substituted for γ IVS 2 in the context of the structural γ globin gene, there is a decrease in transcript accumulation when RNA from K562 cells stably transfected with this gene and grown in hemin is analyzed. This effect of IVS is not unique to K562 cells because the substitution of δ IVS 2 for β IVS 2 has been shown to influence β globin gene induction in MELC, an adult erythroleukemia cell line. In this instance, the substitution of δ IVS 2 for β IVS 2 precludes the ability of a β globin gene to be induced in MELC after growth of the cells in dimethylsulfoxide (DMSO), a compound that causes terminal differentiation and induction of endogenous globin genes in these cells.

In the studies reported here, the substitution of γ IVS 2 for β IVS 2 in γβΔ3' to give γβγ2Δ3' (Fig 1) allows this gene to be induced by hemin stimulation, and suggests that γ IVS 2 may play a role in the regulated expression of γ genes. In addition, we show here that a fusion gene
containing β IVS 2 and the β 3' enhancer, γβ, is both expressed and induced in K562 cells. By contrast, when the β 3' enhancer element is deleted (in the construct γβΔ3'), this gene is expressed but is no longer appropriately induced with hemin. The results in this report and those previously reported\textsuperscript{11,12} suggest that the γ promoter alone is sufficient to direct the expression of γβ fusion genes but is insufficient for induction. When the 3' β enhancer is included in the construct, inducible expression is restored. Similar results are obtained using γneo\textsuperscript{8} with a β 3' enhancer, indicating that internal structural globin gene sequences are not required to mediate this effect. The β 3' enhancer element is tissue-specific because it functions only in erythroid cells; however, our studies indicate that it is not developmental stage-specific because it is active in both embryonic-fetal and adult erythroid cells.

It would be of interest to determine whether the level at which γ IVS 2 and the β 3' enhancer affect induction are similar. The mechanism whereby IVS 2 affects induction has not been resolved. It is most likely that γ IVS alters the kinetics of splicing; however, it is possible that it exerts its inductive effect at the level of transcription initiation perhaps due to an enhancer element within γ IVS 2. It is possible that protein factors binding to γ IVS 2 and not β IVS 2 are involved in this effect. The presence of a β globin gene enhancer in β IVS 2 suggests this possible effect.\textsuperscript{19,21} The β 3' enhancer most likely enhances the expression of the human γ globin gene at the level of transcription initiation. Thus, the mechanisms by which γ IVS 2 and the 3' β enhancer exert their effects may be different and may be complementary to each other in vivo.

“Run on” transcription experiments could distinguish effects on gene transcription suspected for 3' β enhancer regulation from RNA stability and processing activity suggested by effects of γ IVS 2. However, it is difficult to perform meaningful “run on” transcription experiments in K562 cells transfected with γβyγΔ3' because endogenous δ globin gene transcription in these cells would provide transcripts that would hybridize to β IVS 1 probes, and transcripts made from the endogenous γ globin gene would hybridize to γ IVS 2 probes.

In other experiments in this and other laboratories, it has been observed that there are protein factors that bind to both the human γ 5' region and the β 3' enhancer element.\textsuperscript{27-30} In the chicken globin system, embryonic and adult globin genes share a common enhancer element that resides between these two genes.\textsuperscript{31} The binding of protein factors to promoter and enhancer elements could facilitate an interaction between these sequences even when located large distances apart, perhaps by causing a perturbation in chromatin, and as a consequence activate the transcription of the gene whose promoter is brought into proximity of the enhancer. This looping-out model has been proposed as the mechanism for developmental regulation of globin gene expression in the chicken.\textsuperscript{31} In this system, competition of the embryonic and adult gene promoters together with DNA binding proteins bound to these regions for the common enhancer determines which gene is expressed.

In the human system, the presence of a γ enhancer element located 3' to the "γ gene has been suggested on the basis of an increase in chloramphenicol acetyl transferase (CAT) gene activity in transient expression systems.\textsuperscript{32} However, no data using stable transfections with this sequence have been reported. Our data indicate that the β 3' enhancer confers appropriately regulated expression on both γ and β globin genes, and precludes the need for a separate γ enhancer.

We would like to suggest that a model similar to that proposed for the developmental regulation of chicken globin gene expression\textsuperscript{30} is involved in the developmental regulation of human globin gene expression (Fig 4). In this model, there are developmental stage-specific protein factors that determine the equilibrium between the association of the β 3' enhancer with either the γ promoter or the β promoter. In the chicken system, these protein factors interact specifically with nucleotide sequences designated stage selector DNA elements (SSE). In fetal erythroid cells, we suggest that there is at least one γ gene-specific protein factor binding to a γ gene SSE in the γ promoter or other...
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regions 5' to the promoter. The binding of this γ factor to the γ 5' sequences facilitates the interaction of the β 3' enhancer with the γ promoter. In this situation, the γ gene is looped out and transcribed (Fig 4). By contrast, in adult erythroid cells, specific factors binding to the β gene SSE are synthesized and the γ-specific factors diminish or disappear. The binding of the β-specific factors to the β SSE, again presumably in the promoter or 5' to it, facilitates a preferential interaction of the β promoter with the β 3' enhancer; the β gene is looped out and β transcription occurs (Fig 4).

Our data are consistent with such a model. In embryonic-fetal cells, the γ promoter is required for appropriate initiation of transcription. In addition, the inclusion of the β

3' enhancer region contained within a 250-bp Pst I-Pst I fragment located 3' to the β globin polyadenylation site is required for inducible expression of fusion genes containing the γ promoter. Analysis of γneo fusion gene transcription and induction in K562 cells also shows that while the γ promoter is able to direct the tissue-specific expression of the neoγ gene, the β 3' enhancer is required to confer inducible expression on this hybrid gene.

These experiments suggest that the γ promoter is required for developmental stage-specific expression of γ genes in embryonic-fetal cells. The β promoter will not function in these cells, but is required for β globin gene expression in adult erythroid cells.18-21 The β 3' enhancer allows both γβ gene and γneo transcripts to be induced in response to hemin induction. The β 3' enhancer is tissue-specific but is not developmental stage-specific. The β 3' enhancer confers inducible expression on fusion genes containing either the γ or β promoters. In addition, the effect of the enhancer does not require structural globin gene sequences 3' to the γ or β globin promoters to function.

A region upstream of the ε gene referred to as the locus-activating region (LAR) or domain-controlling region (DCR) has been described.3,34 This LAR DNA sequence when linked directly to a human β globin gene and transferred into transgenic mice and tissue culture cells leads to chromosome location-independent and wild-type expression of the β globin gene.3,34 A similar effect has also been recently shown with the γ globin gene.35 In addition, since this paper was submitted, induction of transfected γ fusion gene expression by addition of the LAR has been demonstrated.36 The precise role of this element on developmental- or stage-specific activation of the human β-like globin genes is presently unknown. Further analysis of the interaction of factors binding to the genes in the β globin locus including the ε, γ, δ, and β genes and the LAR during expression in erythropoietic development is required to define the molecular events that regulate this system more precisely; more specifically, the role of interactions between the 5' and 3' elements surrounding these genes, IVS, and the LAR must be understood.

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**Fig 4.** Model for the developmental stage specificity of globin gene expression. The human globin gene cluster is represented on the top line showing the 5' and 3' genes (A), the β gene (B), and the 5' γ gene (C). The promoters of the γ and β genes are indicated by P and the 3' enhancer element is designated by an E. The interaction of developmental stage-specific factors (●) and tissue-specific factors (○) could bring enhancer elements into contact with promoter elements, thereby conferring transcriptional activation on individual globin genes positioned between the promoter and the 3' β enhancer. In fetal cells, this result would occur for the γ genes (A). In adult cells, this would occur for the β globin gene (B).
AN: A ß globin gene, inactive in the K562 leukemic cell, functions normally in a heterologous expression system. Proc Natl Acad Sci USA 81:4485, 1984
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