We have constructed fusion genes comprised of \( \gamma \) and \( \beta \) 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 \( \gamma \) promoter is required for proper initiation of transcription. However, the accumulation of \( \gamma \) globin transcripts in response to hemin induction requires the additional presence of either \( \gamma \) intervening sequence 2 or the \( \beta' \) enhancer element of the \( \beta \) globin gene. Thus, the \( \gamma \) promoter may provide the elements for developmental stage-specific gene expression during fetal life. By contrast, the \( \beta' \) enhancer is erythroid-specific but not developmental stage- or gene-specific.

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**MATERIALS AND METHODS**

**Fusion gene constructs.** The fusion gene constructs are shown in Fig 1. The top line shows a schematic representation of the \( \beta \) globin gene and the important regulatory sequences are indicated. The \( \gamma \) globin 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). The \( \beta' \) globin promoter \( (\gamma' \beta) \) contains the \( \gamma' 5' \) EcoRI-NcoI fragment of 1.3 kilobases (kb) fused to the \( \beta' \) globin gene Neo 1-5'-flanking region of 3.4 kb that includes the \( \beta' \) enhancer. This \( \beta' \) enhancer element was deleted in \( \gamma' \) promoter \( \beta' \Delta 3' \) \( (\gamma' \beta' \Delta 3' \) by removing an Acc 1 EcoRV fragment of 1.3 kb from the \( \beta' \) flanking region. \( \gamma' \) promoter \( \gamma' \) IVS 2-5'-flanking region. \( \gamma' \) promoter \( \gamma' \) IVS 2-5'-flanking region. \( \gamma' \) promoter \( \gamma' \) IVS 2-5'-flanking region. \( \gamma' \) promoter \( \gamma' \) IVS 2-5'-flanking region. \( \gamma' \) promoter \( \gamma' \) IVS 2-5'-flanking region. \( \gamma' \) promoter \( \gamma' \) IVS 2-5'-flanking region.

The \( \gamma' \) promoter neo fusion gene \( (\gamma' neo) \) was made by substituting the 1.3-kb EcoRI-Acy fragment extending from the 5'-region of the \( \gamma' \) gene into the 5'-untranslated region of the SV 40 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 1 (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 genes to evaluate the roles of \( \gamma' \) promoter, \( \gamma' \) IVS 2, \( \beta' \) enhancer, and \( \gamma' \) intragenic sequences on expression and induction in embryonic-fetal erythroid cells. Our results indicate that either \( \gamma' \) IVS 2 or the \( \beta' \) enhancer is required for induction of \( \gamma' \) globin gene expression in K562 cells.

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EcoRI/I ClaI fragment was replaced with the γ gene 5' EcoRI/Acy I fragment. The β 3' enhancer contained within an EcoRI-Bgl II fragment was subcloned downstream of the yneo8 fusion gene in both orientations to generate γneo8 3' β (Fig 1).

Vectors, transfection, and selection of stable transformants. The fusion γβ globin genes were subcloned into pSV2neo,22 a plasmid that contains the neo8 gene transcribed using an SV 40 promoter. Transcription of this gene in eukaryotic cells confers resistance to a neomycin analogue. K562 cells were grown in RPMI 1640 medium (GIBCO) supplemented with 10% fetal calf serum (FCS; Flow Laboratories, Irvine, CA). 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 γβγ2Δ3'. 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 11,12,17; this result is probably because of the integration of transfected genes into different chromosomal positions that lead to variable inducibility of the transfected genes. By contrast, when γ IVS 2 or the β 3' enhancer is absent from constructs, less than 10% of the clones are inducible, a suitable negative control.

To test whether structural globin gene sequences are required for the β 3' enhancer to function, this element was subcloned downstream of a γneo8 fusion gene in both orientations to generate γneo8 3' β (Fig 3). The inducible expression of this gene was evaluated using a γneo8 fusion probe complementary to the 5' end of the γneo8 fusion gene. As previously reported,11 the γneo8 fusion gene alone is transcribed after transfection into K562 cells and the transcripts are appropriately initiated, but there is only occasional induction of this fusion gene.11 By contrast, the γneo8 3' β fusion gene containing the β 3' enhancer is inducible in greater than 50% of the clones analyzed (Fig 3). Additional γ neo 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-

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.

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ROLE OF 3' ENHANCER IN γ GENE EXPRESSION

Fig 2. RNAse protection analysis of transcription of \(\gamma p\) 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 \(\gamma p\) containing the 3' β enhancer (Fig 1, 1). (B) RNA from pools of clones transfected with \(\gamma p\betaΔ3\) (Fig 1, 2). (C) The RNA from individual clones transfected with \(\gamma p\gamma2Δ3\) containing the substitution of \(\gamma IVS 2\) (Fig 1, 3).

DISCUSSION

We have analyzed the expression of \(\gamma\) and β globin fusion genes to evaluate the role of the \(\gamma\) promoter, \(\gamma IVS 2\), and β 3' enhancer sequences on the expression and induction of β globin genes in erythroid cells. An effect of the substitution of \(\gamma\) or \(\delta IVS 2\) for β IVS 2 in fusion globin genes has been previously reported. When \(\gamma IVS 2\) 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 \(\gamma IVS 2\) in the context of the structural β 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 \(\delta IVS 2\) 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 \(\delta IVS 2\) 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 \(\gamma IVS 2\) for β IVS 2 in \(\gamma p\betaΔ3\) to give \(\gamma p\gamma2Δ3\) (Fig 1) allows this gene to be induced by hemin stimulation, and suggests that \(\gamma IVS 2\) may play a role in the regulated expression of \(\gamma\) genes. In addition, we show here that a fusion gene
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.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 γβγ2Δ3' 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.27-30 In the chicken globin system, embryonic and adult globin genes share a common enhancer element that resides between these two genes.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.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.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 expression30 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
The role of 3′ enhancer in γ gene expression in human erythroid cell line K562. Science

A preferential interaction of the enhancer with the γ promoter. In this situation, the γ 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. 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. A similar effect has also been recently shown with the γ globin gene. In addition, since this paper was submitted, induction of transacted γ fusion gene expression by addition of the LAR has been demonstrated. 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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The beta globin 3' enhancer element confers regulated expression on the human gamma globin gene in the human embryonic-fetal erythroleukemia cell line K562

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