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Roles of Fetal $^{G}\gamma$-Globin Promoter Elements and the Adult β-Globin 3′ Enhancer in the Stage-Specific Expression of Globin Genes

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The human fetal $^{G}\gamma$-globin and adult β-globin genes are expressed in a tissue- and developmental stage-specific pattern in transgenic mice: the $^{G}\gamma$ gene in embryonic cells and the β gene in fetal and adult erythroid cells. Several of the *cis*-acting DNA sequences thought to be responsible for these patterns of expression are located 5′ to the $^{G}\gamma$-globin gene and 3′ to the β-globin gene. To further define the locations and functional roles of these elements, we examined the effects of 5′ truncations on the expression of the $^{G}\gamma$-globin gene, as well as the ability of $^{G}\gamma$-globin upstream sequences to alter the developmental regulation of a β-globin gene. We found that sequences between −201 and −136 are essential for expression of the $^{G}\gamma$-globin gene, whereas those upstream of −201 have little effect on the level or tissue or stage specificity of $^{G}\gamma$-globin expression. The $^{G}\gamma$-globin upstream sequences from −201 to −136 were, furthermore, capable of activating a linked β-globin gene in embryonic blood cells; however, a $^{G}\gamma$-globin fragment from −383 to −206 was similarly active in this assay, and the complete fragment from −383 to −136 was considerably more active than either of the smaller fragments, suggesting the presence of multiple *cis*-acting elements for embryonic blood cells. Our data also suggested the possibility of a negative regulatory element between −201 and −136. These results are discussed in relation to several DNA elements in the $^{G}\gamma$-globin upstream region, which have been shown to bind nuclear factors in erythroid cells. Finally, we observed that removal of the β-globin 3′-flanking sequences, including the 3′ enhancer, from the $^{G}\gamma$-globin upstream–β-globin hybrid gene resulted in a 25-fold reduction in expression in embryonic blood cells. This suggests that the β-globin 3′ enhancer is potentially active at the embryonic stage and thus cannot be solely responsible for the fetal or adult specificity of the β-globin gene.

The human fetal γ-globin and adult β-globin genes, when introduced with limited amounts of flanking DNA, are expressed with appropriate developmental and tissue specificity in transgenic mice (7, 8, 20, 39). In the mouse, the endogenous adult β-globin genes are expressed in the fetal liver and the adult bone marrow, but not in the embryonic yolk sac-derived erythroid cells, and human β-globin transgenes show a similar pattern of developmental regulation (8, 24, 39). In contrast, human fetal γ-globin transgenes behave like the orthologous mouse embryonic βh1-globin gene, which is expressed only in the embryonic erythroid cells (7, 20). This has permitted a series of studies in which some of the *cis*-acting DNA sequences responsible for hemoglobin switching have been identified by virtue of their ability to confer stage-specific expression in transgenic mice. Sequences necessary for expression in embryonic blood cells have been localized to the 5′-flanking or 5′ untranslated regions of the $^{G}\gamma$-globin gene, through the analysis of hybrid γ/β-globin genes (41). By similar means, sequences necessary for expression in fetal and adult erythroid cells have been shown to include two enhancers, one located within the β-globin gene (4) and one in the 3′-flanking region (4, 19, 40). In addition to these closely linked control regions, regulatory elements associated with DNase I-hypersensitive sites upstream from the β-globin gene cluster (42) are essential for high-level expression of the β-globin gene (14, 37, 44), although their possible role in hemoglobin switching remains to be determined.

In this study, we investigate in more detail the location and characteristics of the regulatory elements responsible for globin gene expression in mouse embryonic blood cells. We focus primarily on the 5′-flanking region of the human $^{G}\gamma$-globin gene, which our earlier studies have shown to be essential (41). This region is also believed to be critical for γ-globin regulation during human erythroid development, based on gene transfer experiments in human K562 cells (a cell line that expresses γ-globin but not β-globin [1, 18, 22]), as well as the occurrence of point mutations associated with hereditary persistence of fetal hemoglobin (reviewed in reference 34). In one experiment, we analyze the expression in transgenic mice of a series of $^{G}\gamma$-globin genes with progressively truncated 5′-flanking DNA. The results indicate that sequences between positions −136 and −201 upstream from the $^{G}\gamma$-globin cap site contain essential positive elements for expression in embryonic blood cells and possibly negative elements as well. The potential roles of several sequence motifs, known to bind nuclear factors in vitro, are discussed. In a second experiment, we demonstrate that sequences in the same −136 to −201 region, as well as sequences between −201 and −383, can activate transcription from an adjacent β-globin promoter in embryonic blood cells. Finally, we provide evidence that the β-globin 3′-flanking enhancer is capable of stimulating transcription from the β-globin promoter in mouse embryonic blood cells, in the presence of γ-globin upstream sequences. We discuss the implications of this result for the role of the β-globin 3′ enhancer in hemoglobin switching.

MATERIALS AND METHODS

DNA constructs microinjected. Truncated 5′ human $^{G}\gamma$-globin genes used in microinjection were isolated as follows: −383 $^{G}\gamma$-globin gene, 2.57-kilobase (kb) *Stu*I-*Sph*I fragment; −201 $^{G}\gamma$-globin gene, 2.39-kb *Apa*I-*Sph*I fragment; −161 $^{G}\gamma$-globin gene, 2.33-kb *Xmn*I-*Xmn*I fragment; −136 $^{G}\gamma$-

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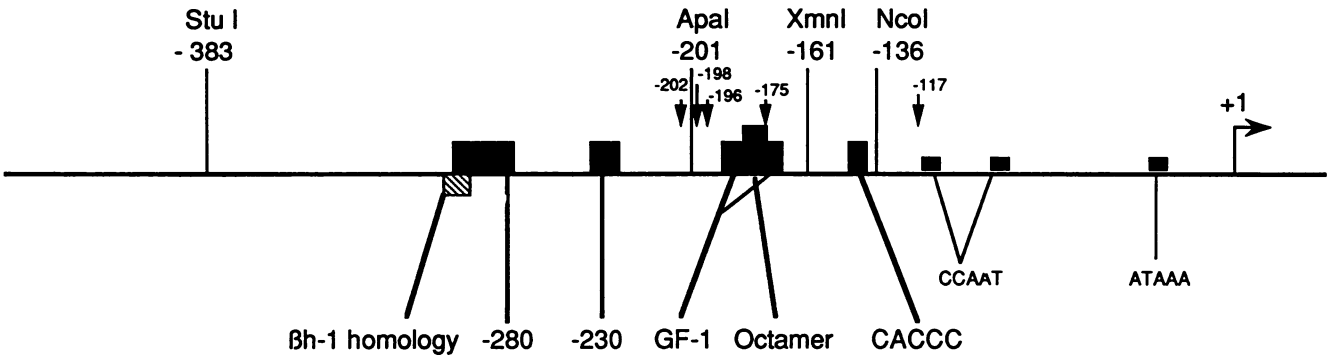

FIG. 1. Human fetal $^{G}\gamma$-globin upstream promoter elements and HPFH point mutants. The positions of restriction enzyme sites used in the 5′-truncated transgenes, relative to the transcription start site (+1), are indicated above the line. Symbols: ■, promoter elements that have been reported to bind nuclear factors (see text); ↓, HPFH point mutants and their positions; ▨, 12-base-pair polypurine sequence (−282 to −293) that is identical to a mouse βh1 embryonic globin upstream sequence.

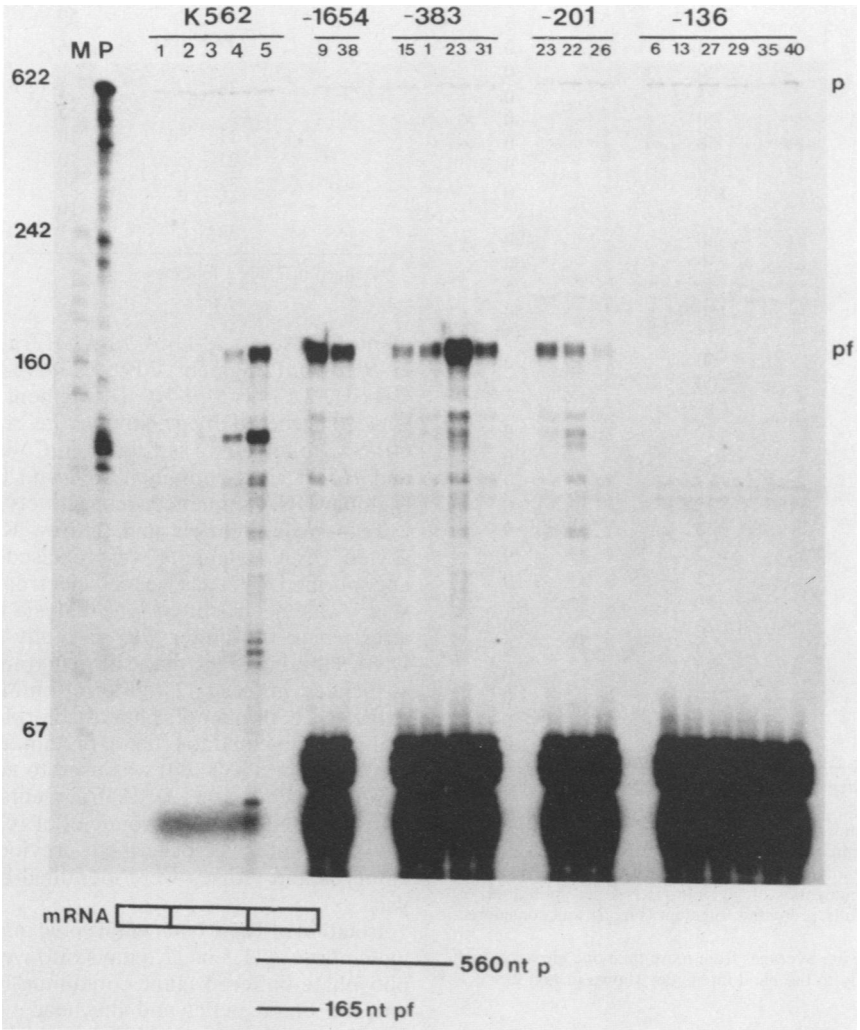

FIG. 2. Expression of 5′-truncated $^{G}\gamma$-globin transgenes measured by RNase protection. The RNA probe specific for the 3′ end of γ-globin mRNA, diagrammed at the bottom (560 nt p) has been previously described (7). p indicates the position of undigested probe (560 nt), and pf indicates the position of the specific 165-nt fragment protected by γ-globin mRNA. Lane M contains ^{32}P-labeled size markers (pBR322 digested with *Hpa*II), some of whose sizes are indicated at the left. Lane P contains undigested RNA probe. Lanes K562 1 to 5 contain 0, 0.03, 0.15, 0.75, and 3 μg of total RNA from hemin-induced K562 cells. The remaining lanes show the analysis of 2 μg each of mouse embryonic blood RNA from selected 11.5-day embryos, carrying the −1654, −383, −201, and −136 truncated genes. Cross-hybridization with endogenous mouse embryonic globin mRNA results in the low-molecular-size fragments seen at the bottom of the gel, which provides an internal control for the amount of RNA used in each hybridization.

TABLE 1. Expression of 5'-truncated $^{G}\gamma$-globin transgenes in embryonic blood

Transgene	Line[a]	No. of DNA copies[b]	Expression[c]
−1654[d]	9	ND[e]	40
	38	ND	12
	28	ND	4
	31	ND	0
	32	ND	0
	36	ND	0
−383	23	80	400
	31	10	10
	1	100	4
	15	60	2
	6	50	0
	22	2	0
	30	10	0
−201[d]	23	100	12
	22	30	8
	26	50	3
	5	3	0
	6	20	0
	12	3	0
	16	10	0
	21	50	0
	40	100	0
	43	150	0
−161	5	300	400
	59	150	200
	8	70	75
	42	150	50
	33	35	35
	26	60	25
	19	100	5
	9	15	3
	2	7	0
	6	3	0
	11	3	0
	40	35	0
	41	300	0
	54	2	0
	60	2	0
−136	27	20	0.4
	6	10	0
	13	20	0
	29	100	0
	35	35	0
	40	10	0

[a] Each line indicates an independent transgenic embryo, listed in decreasing order of expression levels.
[b] DNA copy numbers of individual transgenic lines were estimated from Southern blot analysis by comparison with 1, 10, and 100 copy standards.
[c] Expression levels were estimated by comparison with K562 RNA standards and are expressed in picograms of γ-globin mRNA per microgram of total RNA. Only the 165-nt fully protected fragment (Fig. 2) was considered for the quantitation.
[d] Expression values shown are averages from more than one measurement and do not correspond exactly to the band intensities shown in Fig. 2.
[e] ND, Not determined.

TABLE 2. Expression of 5'-truncated $^{G}\gamma$-globin transgenes in 16.5-day fetal livers

Transgene	Line[a]	No. of DNA copies[a]	Expression[a] in:	
			Liver	Brain
−201	53	5	0	
	57	300	0	
	58	20	0	
	69	100	0	
	75	75	0	
	76	10	0	
	78	400	0	
	82	500	0	
	84	100	0	
	87	50	0	
−161	8	500	0.4	0.4
	43	300	0.1	0.1
	50	50	0.004	
	13	10	0	
	37	100	0	
	40	50	0	
	49	2	0	
−136	38	1	0.25	0.25
	3	5	0	
	11	10	0	
	21	10	0	
	31	50	0	
	32	10	0	
	33	1	0	

[a] Defined in Table 1 footnotes.

globin gene, 2.33-kb *Nco*I (partial)-*Sph*I fragment. Constructs using the *Sph*I site contained 0.8 kb of 3'-flanking DNA, whereas constructs using the *Xmn*I site contained 0.775 kb of 3'-flanking DNA. Hybrid $^{G}\gamma$-globin upstream promoter (−136 to −408)–β-globin plasmid pHL7 was obtained from H. Lin. The constructs used in microinjection were isolated as follows: βB-CX, 3.41-kb *Cla*I-*Xba*I fragment; GB-AX, 3.48-kb *Apa*I-*Xba*I fragment; GB-SsX, 3.60-kb *Sst*I-*Xba*I fragment; GB-SX, 3.66-kb *Stu*I-*Xba*I fragment; GB-SH, 2.53-kb *Stu*I-*Hpa*II fragment. The hybrid GB-SsX was constructed by removing the *Apa*I-*Cla*I fragment of GB-SX, followed by self-ligation. Constructs using the *Xba*I and *Hpa*II sites contained 1.68 and 0.55 kb of 3'-flanking β-globin DNA sequence, respectively.

Transgenic embryos and fetuses. Restriction enzyme-digested DNA fragments were excised from plasmid clones and purified by agarose gel electrophoresis. DNA in gel slices was electroeluted into dialysis bags for 2 to 3 hours, after which the buffer was carefully removed, leaving the DNA adhering to the dialysis membrane. The DNA was then suspended in fresh TE buffer (10 mM Tris, 1 mM EDTA), extracted with phenol, phenol-chloroform, and chloroform, and then subjected to ethanol precipitation. A DNA fluorometer (Hoeffer TKO 100) was used to accurately measure the DNA concentration. DNA fragments (3 to 6 ng/μl) were microinjected into the pronuclei of (C57BL/6J × CBA/J)F_2 mouse zygotes as described previously (17). Transgenic embryos and fetuses were identified by Southern blot analysis.

Isolation of RNA from embryonic and fetal tissues. Individual embryos (11.5 or 12.5 days old) were bled in 3 ml of cold phosphate-buffered saline containing 10 U of heparin per ml, and the blood pellet and the head were stored at −70°C. Livers and brains from 16.5-day-old transgenic fetuses were isolated as described previously (40). RNA was isolated by the LiCl-urea method of Auffray and Rougeon (3).

Analysis of globin mRNA levels by RNase protection. $^{G}\gamma$-globin and β-globin mRNAs were measured with ^{32}P-labeled Sp6 polymerase-synthesized antisense RNA probes (29) spanning either the 5' or 3' end of $^{G}\gamma$-globin or β-globin mRNA. From 2 to 10 μg of total RNA was hybridized at

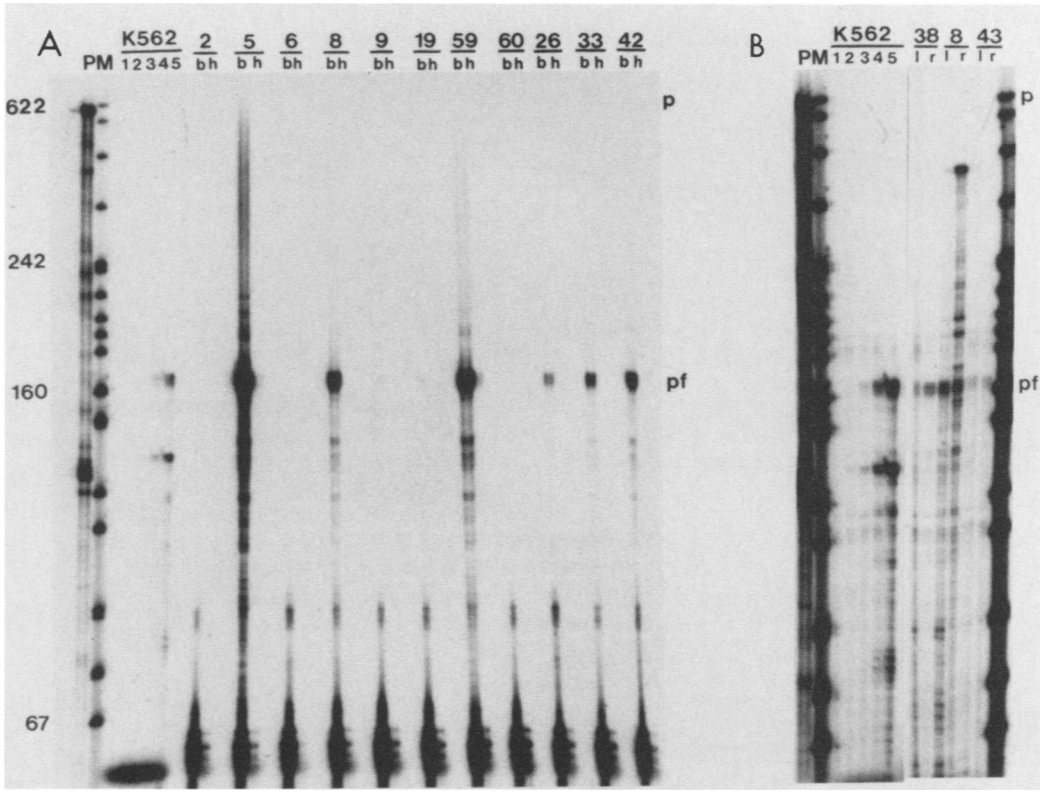

FIG. 3. (A) Expression of the −161 5′-truncated $^{G}\gamma$-globin transgene in mouse embryonic blood. Lane P contains undigested probe (see the legend to Fig. 2). Lane M contains molecular size markers (see the legend to Fig. 2). A 1-μg sample of total RNA from embryonic blood (lanes b) and head (lanes h) of the indicated transgenic embryos (at 11.5 or 12.5 days of gestation) was analyzed by RNase protection. Low-molecular-size bands (60 to 65 nt) derive from cross-hybridization to endogenous mouse embryonic globin mRNAs and provide an internal control for the amount of RNA used in each hybridization. (B) Expression of the −161 and −136 truncated $^{G}\gamma$-globin transgenes in mouse fetal livers. Total RNA (50 μg) from 16.5-day-old fetal liver (l) and brain (r) was analyzed. Fetuses 8 and 43 carry the −161 transgene, whereas fetus 38 carries the −136 transgene. Probe (P), molecular size markers (M), the protected fragment (pf), and K562 RNA positive controls are as described in the legend to Fig. 2.

47°C for 16 to 20 hours and then digested with RNase A and RNase T_1 for 1 h at 15°C. RNase digestion products were analyzed by electrophoresis on polyacrylamide-urea gels and autoradiography. The levels of human globin mRNAs in mouse tissues were estimated by comparison with the hybridization signals obtained with various amounts of hemin-induced human K562 cell RNA (ca. 16 pg of γ-globin mRNA per μg of total RNA [7]) or human adult reticulocyte RNA (10 pg of β-globin mRNA per ng of total RNA [8]) obtained from M. Donovan-Peluso.

RESULTS

Expression of the human $^{G}\gamma$-globin gene with truncated 5′-flanking sequences in mouse embryonic blood cells. In previous experiments, we have shown that the human $^{G}\gamma$-globin gene and several hybrid 5′$^{G}\gamma$/3′β-globin genes, all of which included 1,654 nucleotides (nt) of 5′-flanking DNA, were active in transgenic mouse embryonic blood cells (7, 41). To localize the sequence elements in this 5′ region that are necessary for expression, we first constructed a series of $^{G}\gamma$-globin genes containing 383, 201, and 136 nt of 5′-flanking DNA. Figure 1 shows the restriction enzyme sites used to construct these truncated genes, as well as several conserved sequence elements, a number of sites known to bind nuclear factors, and the positions of point mutations associated with the HPFH phenotype in humans. Each construct was microinjected into fertilized mouse eggs, transgenic embryos were identified at 11.5 days of gestation, blood RNA was isolated, and the levels of $^{G}\gamma$-globin mRNA were measured by an RNase protection assay (Fig. 2; Table 1).

For each construct tested, the expression levels varied widely among different embryos (Table 1, see Fig. 6A), as is often observed in mice carrying globin transgenes (7, 8, 20, 40, 41). We found that the −201 and −383 $^{G}\gamma$-globin transgenes showed a similar range of expression levels to the −1654 transgene tested previously (7), suggesting that no major positive regulatory elements are located upstream of −201. Truncation to −136, however, essentially abolished expression of the $^{G}\gamma$-globin gene. Thus, major positive *cis*-acting elements for expression in mouse embryonic blood cells appear to be located between −136 and −201.

This region contains an octamer element (11, 32), a binding site for the erythroid-specific factor GF-1 (15, 27, 28, 35, 43), and the conserved CACCC box shared by all β-like globin genes (30, 33). To determine whether either the octamer or the GF-1-binding site is essential for tissue-specific expression, we next tested a $^{G}\gamma$-globin transgene truncated at position −161 and thus lacking both of these elements, but retaining the CACCC box (Fig. 1). The −161 transgene was expressed at very high levels in embryonic blood cells (Table 1; see Fig. 6A) and at undetectable or very

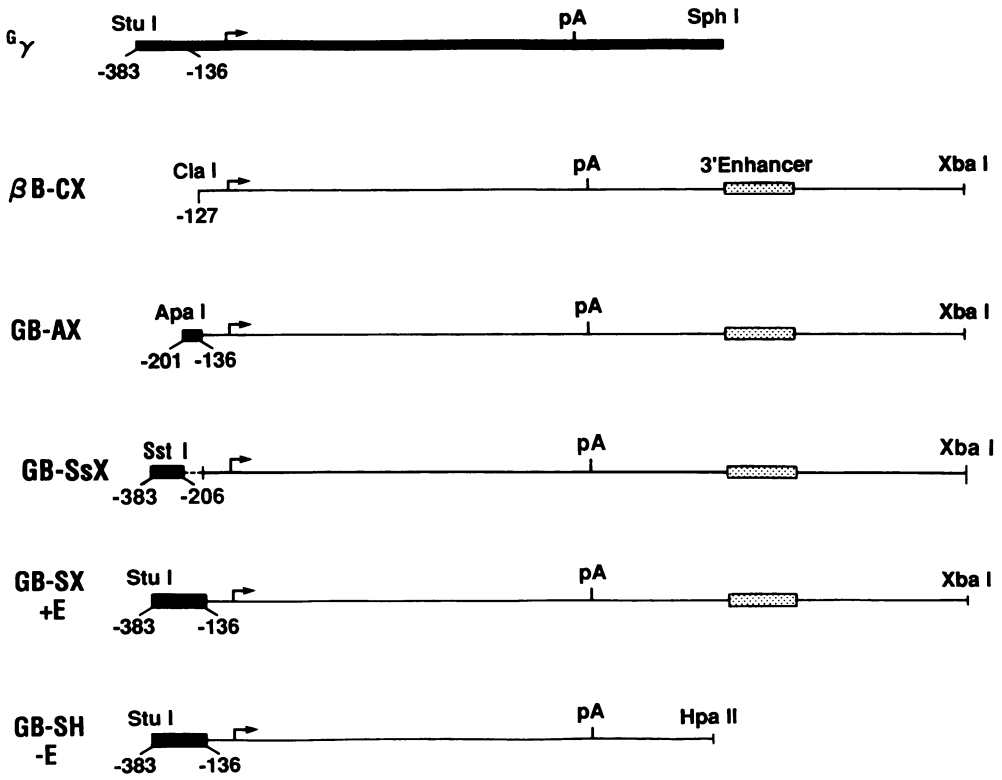

FIG. 4. Hybrid $^{G}\gamma$-globin upstream/β-globin transgenes. Symbols: ■, γ-globin sequences; ——, β-globin sequences; ▩, β-globin 3′ enhancer. Restriction enzyme sites used in the isolation of DNA fragments for microinjection are as indicated. The arrow represents the transcription start site, and pA represents the polyadenylation site for β-globin mRNA.

low levels in the embryonic heads (Fig. 3), which include several nonerythroid tissues. This indicates that sequences in the region between −201 and −161 are not required for tissue-specific expression.

Truncation of $^{G}\gamma$-globin 5′-flanking sequences permits low-level expression in the mouse fetal liver. The occurrence of point mutations in the γ-globin promoter region in cases of nondeletion HPFH has suggested the presence of negative regulatory elements involved in the developmental inactivation of γ-globin gene expression. To determine whether removal of such elements might interfere with the ability of γ-globin transgenes to be switched off at the appropriate stage of mouse development, we examined the expression of several of the 5′-truncated $^{G}\gamma$-globin genes in 16-day-old mouse fetal livers. The −201 transgene was not detectably expressed in fetal livers (Table 2), like the −1654 transgene examined previously (7). In contrast, three of seven fetuses carrying the −161 transgene and one of seven carrying the −136 transgene expressed $^{G}\gamma$-globin mRNA at very low levels in fetal liver as well as brain (Table 2; Fig. 3). This is consistent with the hypothesis that negative regulatory elements in the −161 to −201 region play a role in the inactivation of the $^{G}\gamma$-globin transgene at the fetal stage.

Activation of the adult β-globin gene in embryonic blood cells by $^{G}\gamma$-globin 5′-flanking sequences. We have previously shown that a hybrid gene containing $^{G}\gamma$-globin 5′-flanking DNA (up to −1654), promoter, and 5′-untranslated region, fused to the β-globin structural gene and 3′-flanking sequences, was expressed in transgenic mouse embryonic blood cells (41). To better define the $^{G}\gamma$-globin sequence elements responsible for activation of the hybrid globin transgene, we tested the ability of several $^{G}\gamma$-globin upstream DNA fragments to activate a complete β-globin gene including the β-globin promoter and 127 base pairs of 5′-flanking DNA. The constructs shown in Fig. 4 were microinjected into mouse eggs and expression in blood cells of 11.5-day transgenic embryos was analyzed as described above (Fig. 5). To compare the expression levels of different constructs, we calculated an average level of expression for each by dividing the sum of mRNA levels in all transgenic embryos (including those in which no mRNA was detected) by the total number of embryos.

Although the −127 β-globin transgene (B-CX) was not expressed at significant levels, the addition of $^{G}\gamma$-globin upstream sequences (from −383 to −136; construct GB-SX) resulted in very high levels of expression (Fig. 5 and 6B; Table 3). This agrees with the results of Lin et al. (22), who observed expression of a similar construct in human K562 cells. Thus, γ-globin upstream elements are able to alter the developmental regulation of transcription from the β-globin promoter.

Because the 5′ truncation analysis of the $^{G}\gamma$-globin gene indicated that essential positive elements are located in the −201 to −136 region, we tested the ability of this fragment to activate the −127 β-globin gene (construct GB-AX). Expression in embryonic blood cells was observed, although at much lower levels than with the entire −383 to −136 fragment (Fig. 5 and 6B; Table 3). This suggested the presence of additional positive elements in the −383 to −201 region. Indeed, the −383 to −201 fragment was independently capable of activating the β-globin gene (construct GB-SsX), although again at much lower levels than the −383 to −136 fragment (Fig. 5 and 6B).

The β-globin 3′ enhancer is active in mouse embryonic blood cells. The hybrid globin construct GB-SX was expressed at extremely high levels in mouse embryonic blood

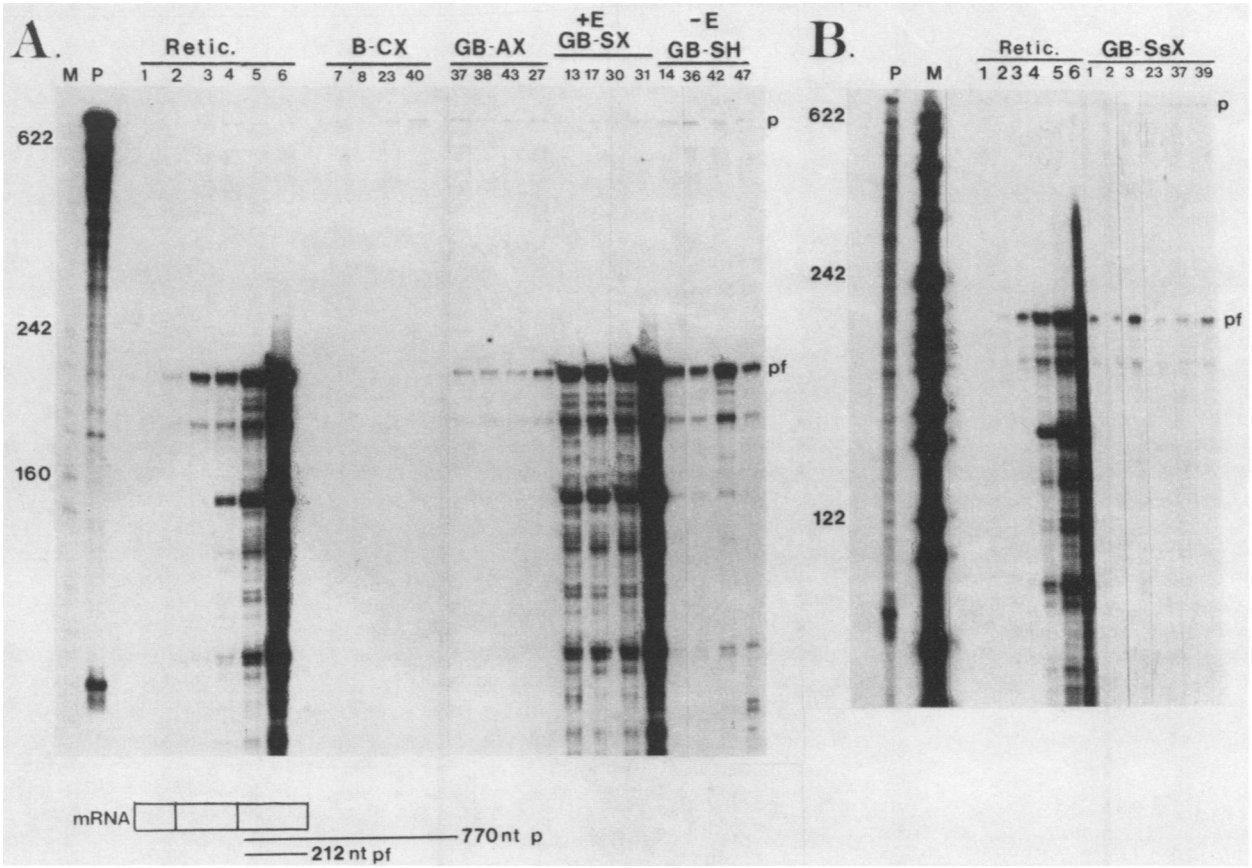

FIG. 5. (A) Expression of hybrid γ-globin upstream/β-globin transgenes in 11.5-day embryonic blood measured by RNase protection. Lanes labeled Retic. 1 to Retic. 6 contain 0, 0.23, 1.1, 5.7, 23, and 230 ng of human reticulocyte total RNA, respectively. The remaining lanes contain 2 μg of total blood RNA from selected transgenic embryos carrying the indicated constructs. The β-globin 3′ RNA probe (p) and protected fragment (pf) are indicated in the diagram at bottom and have been previously described (24). (B) Expression of the GB-SsX hybrid transgene in blood of selected 11.5-day-old embryos. Lanes labeled Retic. are as described for panel A. Total RNA (2.5 μg) from the indicated embryos was analyzed as described for panel A.

cells, compared with $^{G}\gamma$-globin transgenes (compare Tables 1 and 3). This suggested that although the β-globin gene is normally inactive in these cells, certain β-globin sequences must be capable of stimulating the expression of this hybrid gene. To test whether the β-globin 3′ enhancer had any effect, we analyzed the expression of a construct identical to GB-SX but lacking the 3′-terminal 1.1 kb of β-globin-flanking DNA, which includes the 3′ enhancer (GB-SH). Truncation of the β-globin 3′-flanking DNA resulted in a 25-fold reduction in the average level of expression in embryonic blood cells (Table 3; Fig. 6B). Therefore, the β-globin 3′ enhancer appears to be capable of functioning in mouse embryonic as well as fetal and adult erythroid cells.

DISCUSSION

In this study, we have used expression in transgenic mice as an assay to characterize DNA sequences involved in the developmental stage-specific expression of human γ- and β-globin genes. We first focused on the 5′-flanking region of the $^{G}\gamma$-globin gene and used two different approaches to define regulatory sequences in this region: (i) we analyzed the effects of 5′ truncations on the expression of the $^{G}\gamma$-globin gene; and (ii) we tested the ability of $^{G}\gamma$-globin 5′-flanking DNA fragments to activate a β-globin gene in embryonic erythroid cells. Together, these studies identify two regions containing positive elements for expression in mouse embryonic blood cells. Sequences between −201 and −136 are required for efficient expression of the $^{G}\gamma$-globin gene, and, furthermore, they are able to activate the β-globin gene in *cis*, although at a low level. Sequences between −383 and −201, although not essential for expression of the $^{G}\gamma$-globin gene, are capable of activating the β-globin gene at similar levels to the −201 to −136 fragment. A larger fragment containing both of these regions (−383 to −136) was considerably more effective at activating the β-globin gene than either of the two smaller fragments. Our data also provided evidence of negative regulatory elements between −201 and −161: removal of these sequences from the $^{G}\gamma$-globin gene resulted in a low level of expression in the fetal liver, in which human $^{G}\gamma$-globin transgenes are normally silent. Finally, we made the surprising observation that the human β-globin 3′ enhancer appears to be active in embryonic blood cells, although the β-globin gene is not normally expressed in these cells.

The upstream $^{G}\gamma$-globin region we have analyzed contains a number of conserved sequences elements, as well as sequence motifs that have been shown to bind nuclear factors present in erythroid cells. Although the constructs we have tested in transgenic mice do not allow us to attribute specific roles to any of these elements, they do allow us to

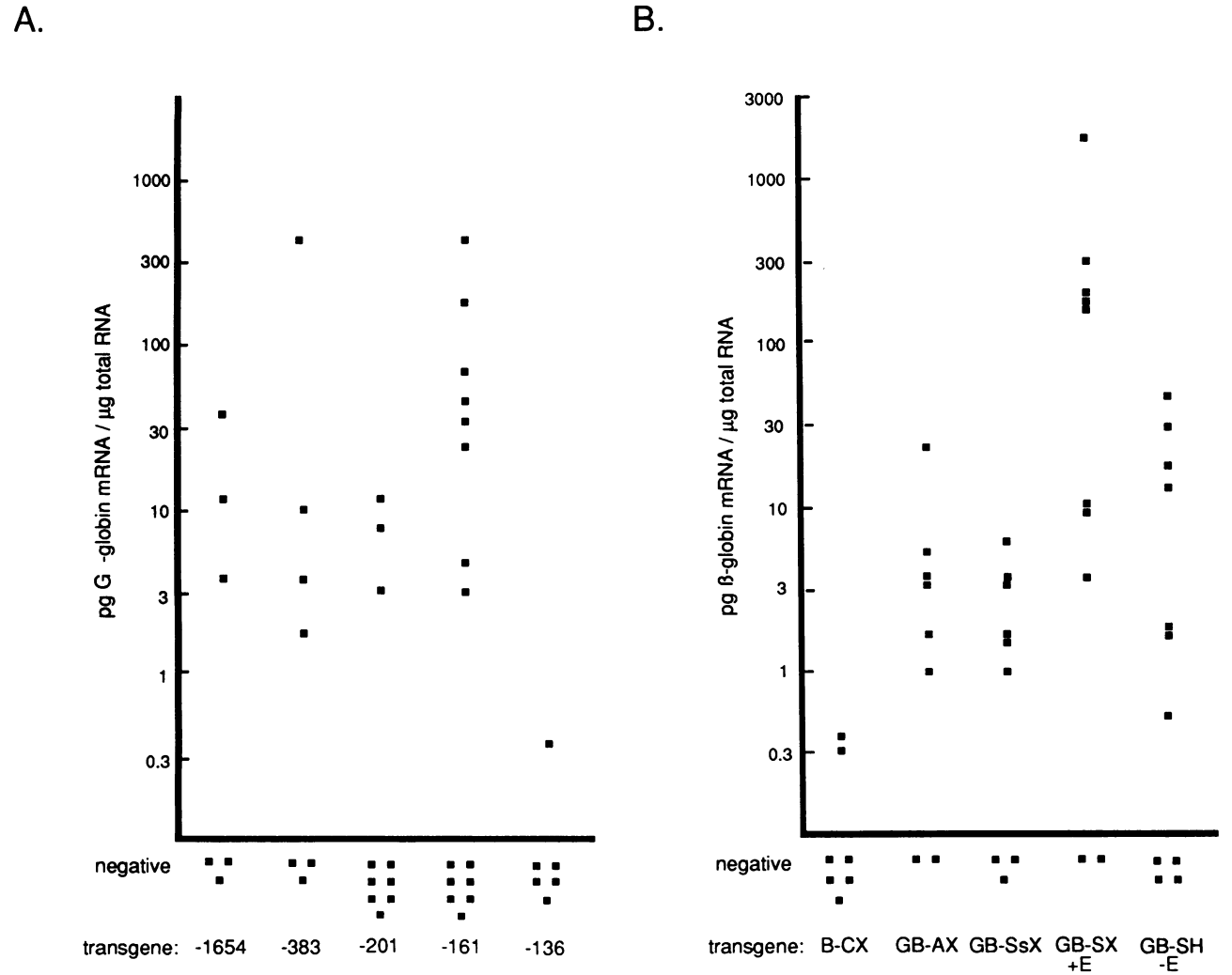

FIG. 6. Comparison of transgenic mRNA expression levels in mouse embryonic blood. The different 5'-truncated $^{G}\gamma$-globin transgenes (A) and the γ-β hybrid transgenes (B) are compared. Each dot indicates the expression level of an independent transgenic embryo (data from Tables 1 and 3). Dots below the abscissa indicate embryos in which no expression was detected.

rule out certain models and to suggest possible roles for some of the elements. The −383 to −201 region contains three sequences of potential interest (Fig. 1): a 12-base-pair polypurine sequence (−282 to −293) that is identical to a sequence (position −286 to −297) upstream from the mouse βh1 embryonic globin gene (16; R. Hardison, personal communication), and two sequences (labeled −280 and −230 in Fig. 1) that are footprinted by nuclear extracts of human K562 cells (6, 23). Our deletion studies indicate that none of these sequences substantially influence the level of expression of the $^{G}\gamma$-globin gene in mouse embryonic blood cells. However, they could be involved in the ability of the −383 to −201 fragment to activate the β-globin gene.

The −201 to −136 region contains a number of sites that have been shown to bind nuclear factors in vitro: an octamer element (5'-ATGCAAAT-3') at positions −175 to −182; two sequences, at positions −171 to −175 and −185 to −195, that bind the erythroid-specific factor GF-1 (15, 26–28, 43, 45); and the CACCC box, present in the 5'-flanking region of all β-like globin genes (30), at positions −139 to −147. Deletion of all three of these elements (−136 truncation) abolished expression of the $^{G}\gamma$-globin transgene in embryonic blood cells, but deletion of only the octamer and GF-1 sites (−161 truncation) had no such effect. This indicates that only the CACCC box, or other elements in the −161 to −136 region, are essential. The CACCC box, which binds a ubiquitous protein in vitro, has been shown to serve as a transcriptional activator of the β-globin and other genes (30, 33). Although other studies have suggested that the octamer and GF-1 sites in the γ-globin promoter are involved in regulation (6, 23, 27, 28), our results demonstrate that neither is required for tissue- and stage-specific expression of the $^{G}\gamma$-globin gene in transgenic mice. Therefore, other sequences within the $^{G}\gamma$-globin gene or in the more proximal promoter region must be sufficient to confer the observed pattern of regulation. These might include several sequences that resemble the consensus sequence for GF-1-binding sites, or other sites for factors yet to be discovered.

The ability of the −383 to −136 $^{G}\gamma$-globin fragment to activate the −127 β-globin gene indicates that this fragment contains the sequences responsible for expression in embryonic blood cells. Presumably, stage-specific erythroid factors interact, directly or indirectly, with sequences on this fragment and thus activate transcription from the linked β-globin promoter, whereas the same factors fail to activate the β-globin promoter in its normal context. GF-1 seems

TABLE 3. Expression of hybrid γ-globin upstream/β-globin transgenes in embryonic blood

Transgene	Line	No. of DNA copies[a]	Expression[a,b]
B-CX	4	200	0.4
	23	50	0.3
	7	20	0
	8	5	0
	11	50	0
	22	5	0
	40	10	0
GB-AX	12	100	25
	27	150	5
	37	70	4
	38	200	4
	43	30	2
	30	10	1
	23	5	0
	28	10	0
GB-SsX	3	150	6
	39	100	3.5
	1	150	3
	2	125	2
	37	30	2
	23	2	1
	11	2	0
	44	10	0
	45	10	0
GB-SX	31	100	2,000
	17	20	300
	13	50	200
	30	20	200
	35	80	200
	11	5	10
	18	5	10
	22	2	4
	27	2	0
	38	2	0
GB-SH	42	80	50
	36	20	30
	47	10	20
	14	50	15
	23	5	1.5
	25	100	1.5
	61	100	0.5
	5	5	0
	24	50	0
	30	30	0
	63	5	0

[a] Defined in Table 1 footnotes.

[b] Averages are as follows: B-CX, 0.1; GB-AX, 5; GB-SsX, 2; GB-SX, 292; GB-SH, 11. They are computed as the sum of all expression values (including nonexpressing embryos) divided by the total number of embryos analyzed.

unlikely to be solely responsible for this effect, because it is present at all stages of erythroid development and interacts with sequences in both the β- and γ-globin genes (15, 26–28, 35, 43, 45). The −383 to −201 fragment and the −201 to −136 fragment are both active in our assay, but each one is 50- to 100-fold less active than the entire −383 to −136 fragment. This suggests either that multiple elements in the two regions act in synergy, or, alternatively, that an important element is disrupted when the fragment is cleaved at the −201 *Apa*I site. The latter possibility is consistent with the occurrence of three independent forms of nondeletion HPFH associated with mutations in this region, at positions −202, −198, and −196 (10, 12, 13, 34, 38). However, no binding sites for nuclear factors in this region have yet been reported.

In addition to positive elements, the presence of a negative regulatory element(s) in the $^{G}\gamma$-globin upstream region was suggested by the observation that several day-16 fetuses carrying the −161 or the −136 $^{G}\gamma$-globin transgenes produced $^{G}\gamma$-globin mRNA in the fetal liver and brain. Although the levels of expression in fetal tissues were extremely low, this result appears to be significant, because no expression was ever detected in a large number of transgenic fetuses carrying the −201 or −1654 $^{G}\gamma$-globin transgene. Thus, the −201 to −161 region may contain a *cis*-acting element involved in the repression of γ-globin expression in mouse fetal erythroid and as well as nonerythroid cells (although the low levels of expression suggest that other negative regulatory mechanisms are unaffected by removal of these sequences). We also observed that the −161 $^{G}\gamma$-globin transgene appeared to be expressed at somewhat higher levels than the −201 transgene in embryonic erythroid cells (Table 1; Fig. 6). Although this difference is certainly not conclusive, it raises the possibility that the −201 to −161 region may also contain a negative element that functions in embryonic erythroid cells.

It is interesting that the octamer element, which is located in this region, may mediate negative transcriptional regulation in certain circumstances. A base substitution at position −175, within the octamer consensus sequence, is associated with a form of human $^{G}\gamma$ HPFH (31, 36). Although the ubiquitous Oct-1 factor has been shown to bind to the normal γ-globin promoter at this site, the −175 mutant promoter fails to bind this factor and also shows increased expression in vivo and in transfection assays. Thus, it has been suggested that the octamer may serve as a negative regulatory element (15, 27, 31, 36), although this interpretation has recently been questioned (28). In addition, a different octamer-binding protein (Oct-3) has been reported to have a negative transcriptional effect on the mouse immunoglobulin heavy-chain gene in undifferentiated embryonal carcinoma cells (21). Whether the octamer or some other element in the −161 to −201 interval may be responsible for the negative effects we observed remains to be investigated.

One surprising result was the finding that the 3′ enhancer of the adult β-globin gene appears to be functional in mouse embryonic blood cells. Deletion of β-globin 3′-flanking sequences including the enhancer, in the presence of $^{G}\gamma$-globin upstream sequences, resulted in a 25-fold reduction in the average level of expression in embryonic blood (construct GB-SX versus GB-SH). By comparison, a similar truncation of 3′-flanking sequences from the β-globin gene caused an approximately 10-fold reduction in β-globin expression in the mouse fetal liver (40). These data suggest that the 3′-flanking enhancer is equally active in mouse embryonic blood cells, where the β-globin gene is not expressed, and in the fetal liver, where the gene is maximally expressed. The β-globin 3′ enhancer has also been recently shown to function in human K562 cells, where it restores the ability of a γ-globin promoter–neomycin resistance fusion gene to be induced by hemin (M. Donovan-Peluso et al., submitted for publication). This apparent lack of developmental specificity is consistent with (although not proven by) the observation that the β-globin 3′ enhancer contains multiple binding sites for the factor GF-1, which is present at all stages of erythroid development (45).

It has been demonstrated that in the chicken β-globin gene complex, one enhancer, situated between the adult β-globin

and the embryonic ε-globin gene, can activate each gene in a different stage-specific pattern, which is determined by promoter elements (9). As the human β-globin 3′ enhancer is situated 25 to 30 kb from the γ-globin promoters, its natural function seems likely to be the activation of only the β-globin gene, not the γ-globin genes. The latter function may be provided by a sequence 3′ to the human ^{A}γ-globin gene, reported to have enhancer activity in transfection experiments in K562 cells (5). However, the immunoglobulin heavy-chain enhancer has been reported to activate a V_H promoter over a distance of 17.5 kb (46), so a role for the β-globin 3′ enhancer in γ-globin expression may be possible.

Our results lead to the conclusion that the fetal or adult stage-specific expression of the β-globin gene must be regulated by elements other than the 3′ enhancer. A requirement for β-globin 5′-flanking sequences upstream of −127 appears to be ruled out, because the −127 β-globin gene was appropriately inactive in embryonic blood cells (Table 3), whereas a −122 β-globin gene was expressed in adult transgenic mouse reticulocytes at similar levels to β-globin transgenes with several kilobases of 5′-flanking DNA (39). Furthermore, a *Bam*HI-*Xba*I fragment of the β-globin gene, containing the 3′ enhancer as well as intron 2 and exon 3, but lacking the β-globin promoter, confers fetal or adult stage specificity to the simian virus 40 early promoter (25). Intragenic sequences within intron 2 and/or exon 3 of the β-globin gene display enhancer activity in transgenic mouse fetal liver (4) and in MEL (2) cells. The potential role of this internal enhancer, or other intragenic elements, in the stage-specific expression of the β-globin gene remains to be explored.

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