Upstream Gγ-Globin and Downstream β-Globin Sequences Required for Stage-Specific Expression in Transgenic Mice

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The human Gγ-globin and β-globin genes are expressed in erythroid cells at different stages of human development, and previous studies have shown that the two cloned genes are also expressed in a differential stage-specific manner in transgenic mice. The Gγ-globin gene is expressed only in murine embryonic erythroid cells, while the β-globin gene is active only at the fetal and adult stages. In this study, we analyzed transgenic mice carrying a series of hybrid genes in which different upstream, intragenic, or downstream sequences were contributed by the β-globin or Gγ-globin gene. We found that hybrid 5'Gγ/3'β globin genes containing Gγ-globin sequences upstream from the initiation codon were expressed in embryonic erythroid cells at levels similar to those of an intact Gγ-globin transgene. In contrast, β-globin upstream sequences were insufficient for expression of 5'β/3'Gγ hybrid globin genes or a β-globin-metallothionein fusion gene in adult erythroid cells. However, β-globin downstream sequences, including 212 base pairs of exon III and 1,900 base pairs of 3'-flanking DNA, were able to activate a 5'Gγ/3'β hybrid globin gene in fetal and adult erythroid cells. These experiments suggest that positive regulatory elements upstream from the Gγ-globin and downstream from the β-globin gene are involved in the differential expression of the two genes during development.

The sequential expression of different members of the β-globin gene family during ontogeny provides an excellent system for the study of developmental gene regulation in mammals. In humans, the first of these genes to be expressed is the ε-globin gene, which is active in the embryonic erythrocytes that arise in the yolk sac during the early weeks of gestation. The Gγ- and Aγ-globin genes become active later in gestation, primarily in fetal liver erythroid cells, and their expression gradually decreases to a very low level after birth. Finally, the adult δ-globin and β-globin genes are expressed at low levels in the fetus and achieve their maximal expression only after birth, as the site of erythropoiesis shifts to the bone marrow (8). The expression of the mouse β-globin gene family differs in that two genes, ϵ^y (homologous to human ε) and βh1 (homologous to human Gγ and Aγ), are expressed in the embryonic erythrocytes, while the other two functional genes, βmaj and βmin, are expressed in both fetal and adult erythroid cells (21).

When cloned human β-globin and γ-globin genes were introduced into the mouse germ line, together with a limited amount of 5'- and 3'-flanking DNA, each of these genes was expressed in a tissue- and stage-specific pattern similar to that of its murine homolog. Thus, the β-globin gene was expressed in fetal and adult but not embryonic erythroid cells (4, 9, 15, 23), while the Gγ- or Aγ-globin gene was expressed only in embryonic erythroid cells (3, 9, 13, 24). These studies indicated that cis-acting regulatory elements able to dictate tissue-specific and stage-specific expression were located close to or within the β- and γ-globin genes and furthermore that transgenic mice could provide an experimental system in which to localize these regulatory elements.

In this study, we have examined various regions of the β- and Gγ-globin genes for the presence of regulatory elements that might be responsible for their differential expression in transgenic mice. We found, first, that sequences 5' to the initiation codon of the Gγ-globin gene are both necessary

and sufficient for the expression of hybrid globin genes in mouse embryonic blood cells. In contrast, β-globin upstream sequences are insufficient for expression in fetal and adult erythroid cells. β-Globin 3' sequences, however, contain regulatory elements that activate transcription from the Gγ-globin promoter in hybrid γ/β-globin genes.

MATERIALS AND METHODS

DNA constructions. Hybrids between the human Gγ-globin gene and the human β-globin gene were produced at three conserved restriction enzyme sites (NcoI, BamHI, and EcoRI) and inserted into pBR322. The Gγ-globin gene contains three NcoI sites near the 5' end of the gene, requiring the use of partial digestion to generate the fusion at the desired site. All three 5'Gγ/3'β hybrids were isolated as BglII-SphI fragments with 1,659 base pairs (bp) of 5'- and 1,895 bp of 3'-flanking DNA. Two of the 5'β/3'Gγ hybrids (NcoI and BamHI) were isolated as SphI-SphI fragments with 614 bp of 5'- and 620 bp of 3'-flanking DNA, while the third hybrid (EcoRI) was isolated as an HincII fragment with 815 bp of 5'- and 611 bp of 3'-flanking DNA.

The β-globin-metallothionein fusion gene (a gift of D. Falb and T. Maniatis) contained mouse βmaj-globin sequences between ClaI (−1.2 kilobases [kb]) and HincII sites (+26) joined to mouse metallothionein I sequences between a BglII site at +65 and an XhoI site at +2200.

Production of transgenic mice. DNA fragments were excised from plasmid clones and purified by agarose gel electrophoresis, followed by centrifugation on CsCl gradients (11). DNA was injected into the pronuclei of (C57BL/6J × CBA/J)F₂ fertilized eggs as described (11). Founder transgenic mice and transgenic progeny were identified by Southern blot analysis of tail DNA.

Isolation of RNA from transgenic mouse tissues. Peripheral blood was obtained from the tail or by heart puncture and bone marrow was obtained from the femurs of adult transgenic mice. Embryonic blood and fetal livers were obtained as described by Chada et al. (3). RNA was isolated

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by the guanidine thiocyanate-CsCl method of Chirgwin et al. (6) or by the LiCl-urea method of Auffray et al. (2). The integrity of all RNA preparations was monitored by electrophoresis on formaldehyde-agarose gels (16).

Analysis of globin mRNA levels by RNase protection. The RNA probes used to measure the levels of γ/β hybrid globin mRNAs are shown in Fig. 2. The 5' γ-globin probe was derived from the Aγ-globin gene but cross-hybridized with Gγ-globin mRNA. β/γ hybrid globin mRNAs were measured with probes for the 5' end of β-globin or the 3' end of Gγ-globin. From 1 to 10 μg of total RNA was hybridized with 4 ng of RNA probe for 16 to 24 h at 45 to 50°C, followed by digestion with RNase A and T_1 (17). The RNase digestion products were analyzed by electrophoresis on polyacrylamide-urea gels and autoradiography. The levels of hybrid globin mRNAs in mouse erythroid tissues were estimated in comparison to hybridization with various amounts of hemin-induced human K562 cell RNA (14) or human adult reticulocyte RNA. The level of human Gγ-globin mRNA in the K562 cell RNA was measured to be 16 pg/μg of total RNA (3), while the level of β-globin mRNA in human adult reticulocyte RNA was 10 pg/ng of total RNA (4).

RESULTS

Experimental design. To localize the *cis*-acting DNA sequences responsible for the differential stage-specific expression of the Gγ- and β-globin genes, we used the six hybrid
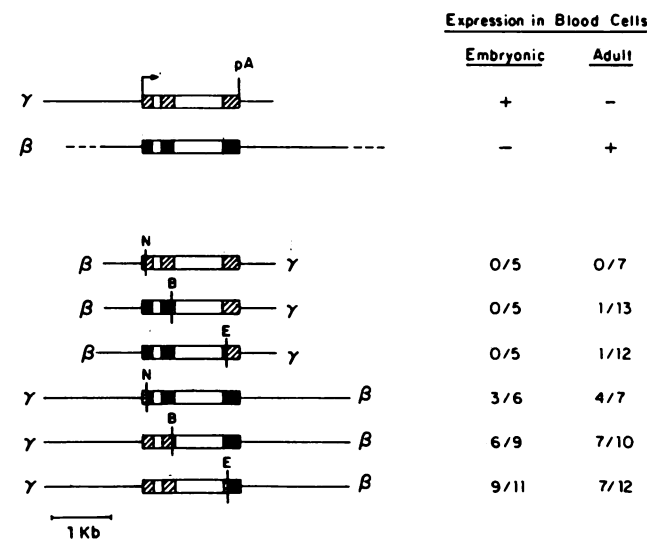

FIG. 1. Hybrid β/γ and γ/β globin genes. The γ-globin gene at top is the Gγ-globin gene fragment (*BglII-SphI*) which was expressed specifically in embryonic blood cells of transgenic mice (3). The β-globin gene, with various amounts of 5'- and 3'-flanking DNA, was expressed in fetal and adult but not embryonic erythroid cells (9, 23). The hybrid genes shown were constructed by fusing the two genes at the *NcoI* (N), *BamHI* (B), or *EcoRI* (E) sites and contained the indicated 5'- and 3'-flanking DNA (see Materials and Methods). The numbers at right show the fraction of transgenic lines that expressed each hybrid gene in 11.5-day-old embryonic blood cells and in adult blood cells. The levels of expression of the γ/β hybrid genes are listed in Table 1. Low-level expression was observed in adult blood as well as brain in one transgenic line carrying the β/γ *BamHI* hybrid gene and in adult blood in one line carrying the β/γ *EcoRI* hybrid gene (in each case, less than 1 pg of mRNA per μg of total RNA). pA, Polyadenylation site. Hatched and solid boxed, Gγ^- and β-globin exons, respectively. Open boxes, Introns.

globin genes shown in Fig. 1. These were constructed by fusing portions of the β- and Gγ-globin genes at three conserved restriction enzyme sites: and *NcoI* site at the initiation codon; a *BamHI* site in the second exon; and an *EcoRI* site in the third exon. Experiments involving gene transfer into murine erythroleukemia (MEL) cells had implicated sequences on both sides of the initiation codon in the regulation of β-globin gene expression (5, 25). For this reason, our hybrid genes were designed to detect possible intragenic or downstream regulatory elements as well as upstream elements. The amount of 5'- and 3'-flanking DNA included in the hybrid constructs was sufficient for tissue- and stage-specific expression of the individual β-globin or Gγ-globin genes in previous studies (3, 23).

The six hybrid genes were microinjected into fertilized mouse eggs. For each hybrid gene, between 7 and 13 transgenic lines were generated, each carrying one or more intact copies of the gene. To analyze the expression of each hybrid gene, we isolated RNA from circulating blood cells of 11.5-day-old transgenic embryos and from bone marrow or peripheral blood (in which most of the RNA derives from reticulocytes) of adult transgenic mice. We examined fetal liver RNA from only a few selected transgenic lines, as mouse fetal and adult erythroid cells are very similar with respect to which globin genes (endogenous or exogenous) are expressed and which are silent (3, 9, 15, 21). The levels of mRNA from the transgenes were measured by RNase protection assays with the probes described in Fig. 2 and Materials and Methods.

Expression of 5'β/3'γ and 5'γ/3'β hybrid globin genes. As indicated in Fig. 1, all three β/γ hybrid genes failed to be expressed in the great majority of transgenic lines. No expression was seen at the embryonic stage in any of the lines examined. Only 1 of 13 lines carrying the *BamHI* hybrid and 1 of 12 lines carrying the *EcoRI* hybrid showed expression in adult blood cells (data not shown), in each case at a low level (Fig. 1).

In contrast, each of the three γ/β hybrid genes was expressed in both embryonic and adult erythroid cells in the majority of transgenic lines (Fig. 1). Examples of the RNase protection assays used to detect the hybrid globin mRNAs are shown in Fig. 2, and the mRNA levels in each transgenic line are listed in Table 1. The γ/β hybrid genes were also expressed in the fetal liver when this stage was examined. Therefore, all three γ/β hybrid genes contained *cis*-acting sequences sufficient for expression in erythroid cells at the embryonic as well as the fetal and adult stages, whereas the three β/γ hybrid genes appeared to lack sequences required for expression at each of these stages.

Comparison of γ/β hybrid genes with intact Gγ- and β-globin genes. For each γ/β hybrid gene, the level of expression at a given stage of development varied considerably between different transgenic lines, as was seen previously with intact β- and Gγ-globin genes (3, 9, 23). Therefore, it was necessary to examine a large number of transgenic lines carrying each of the three hybrid genes to compare their levels of expression and also to compare these with the expression of intact Gγ-globin and β-globin genes (Fig. 3). In embryonic blood cells, the distribution of mRNA levels for the three γ/β hybrid genes did not appear to differ significantly from each other or from the mRNA levels observed with intact γ-globin genes (Fig. 3A). In adult blood cells, there was again no clear difference between the expression levels of the three γ/β hybrid genes. However, all three were expressed at levels approximately one order of magnitude lower than an intact β-globin gene (Fig. 3B).
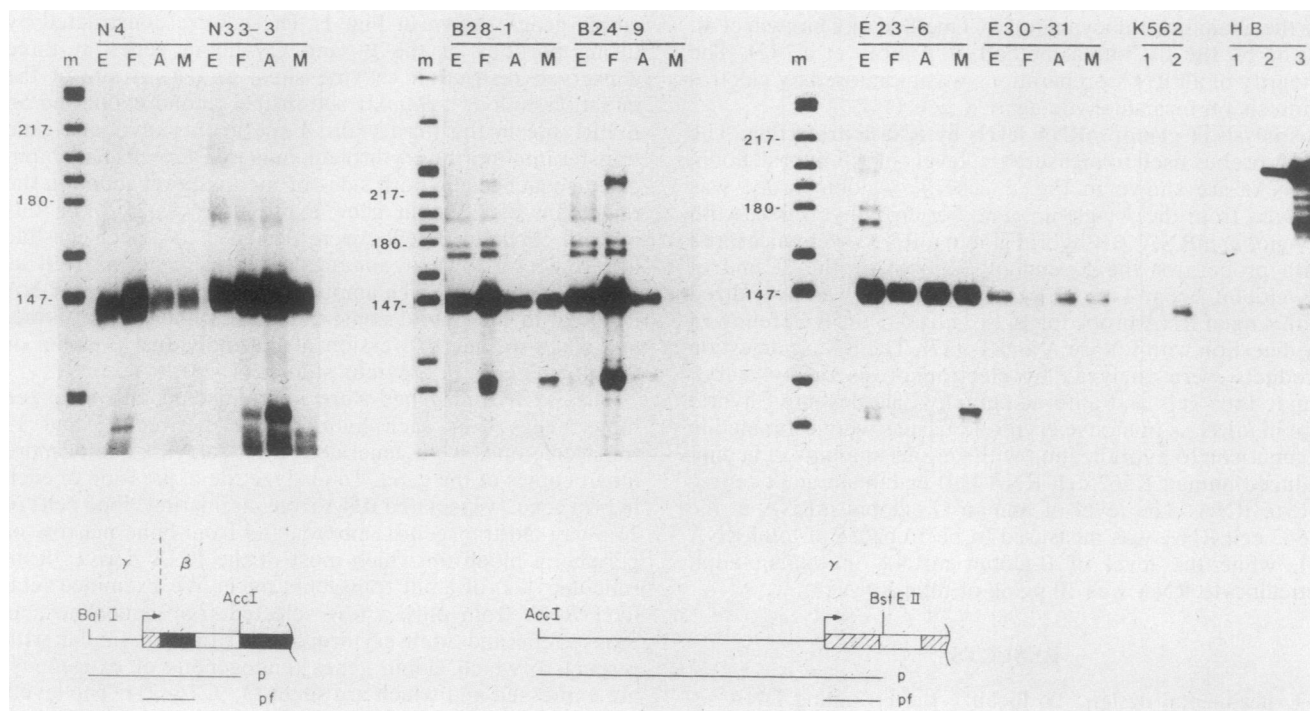
FIG. 2. Expression of γ/β hybrid genes in erythroid tissues of transgenic mice. RNase protection analysis of RNAs from two transgenic lines carrying each of the three γ/β hybrid globin genes is shown. RNAs from mice carrying the NcoI hybrid gene (N4 and N33-3) were analyzed with a 5' probe derived from the same hybrid gene (BalI-AccI fragment), while those from mice carrying BamHI (B28-1 and B24-9) or EcoRI (E23-6 and E30-31) hybrid genes were analyzed with a 5' γ-globin probe. Total RNA (4-μg sample) was analyzed from (E) 11.5-day-old embryonic blood, (F) 16.5- or 17.5-day-old fetal liver, (A) adult peripheral blood, and (M) adult bone marrow. Lanes m, Molecular size markers (in base pairs). In HB, lanes 1, 2, and 3 contain 0.23, 2.3, and 23 ng of human reticulocyte total RNA, respectively, hybridized with an RNA probe for the human β-globin gene (3' EcoRI-PstI fragment). In K562, lanes 1 and 2 contain 0.32 and 1.6 μg of total RNA, respectively, from hemin-induced human K562 cells, hybridized with the 5' γ-globin probe. The two probes were of identical specific activity. p, RNA probes used for analysis of Gγ/β hybrid mRNAs; pf, protected fragment (147 nucleotides). While the K562 and HB standards can be compared with the E23-6 and E30-31 RNAs in this figure, the autoradiograms in other panels were exposed for different lengths of time and cannot be directly compared.

To summarize these results, in mouse embryonic blood cells expression of the Gγ-globin gene and the inactivity of the β-globin gene appeared to be determined by sequences upstream from the initiation codon. Each of the hybrid genes containing upstream Gγ-globin sequences was expressed at levels similar to the intact Gγ-globin gene, while those hybrid genes containing upstream β-globin sequences were not expressed. In contrast, in adult erythroid cells, β-globin upstream sequences were insufficient for expression, and addition of intragenic (NcoI-EcoRI) β-globin sequences failed to overcome this deficiency. However, β-globin sequences 3' to the EcoRI site were able to activate a hybrid γ/β gene in fetal and adult mouse erythroid cells.

β-Globin upstream sequences are insufficient for expression of a β-globin-metallothionein fusion gene. As an independent test of the role of β-globin upstream sequences in expression in adult erythroid cells, we examined the expression of a β-globin-metallothionein fusion gene in transgenic mice. The fusion gene contained sequences from −1.2 kb to +26 bp (relative to the cap site) from the mouse βmaj-globin gene joined to position +65 of the mouse metallothionein I structural gene. Using an RNase protection assay with a probe specific for the fusion mRNA, we were unable to detect any expression in adult blood or bone marrow in five independent transgenic lines (data not shown). This confirmed the conclusion that upstream β-globin sequences are insufficient for expression in adult erythroid cells.

DISCUSSION

The aim of this study was to define regions of the human β-globin and Gγ-globin genes containing the cis-acting regulatory elements responsible for their differential stage-specific expression. Previous experiments had shown that these two genes are expressed in erythroid cells at distinct stages of development in transgenic mice. In this study, we have analyzed the expression in transgenic mice of six different hybrid globin genes derived from the two human genes (Fig. 1), as well as a β-globin-metallothionein fusion gene. Based on these experiments, we may draw several conclusions about the location of regulatory elements associated with the β-globin and Gγ-globin genes.

Gγ-globin sequences between the upstream BglII site (−1.7 kb from the cap site) and the NcoI site (initiation codon) were necessary and sufficient for the expression of hybrid globin genes in mouse embryonic erythroid cells. Replacement of these sequences with β-globin upstream sequences in a β/γ hybrid gene eliminated embryonic expression, while these Gγ-globin sequences alone could activate a γ/β hybrid gene to approximately the same level as an intact Gγ-globin gene. This suggests that sequences upstream from the Gγ-globin gene contain a positive regulatory element(s) that is recognized by a trans-acting factor(s) in mouse embryonic erythroid cells. It is also possible that a negative element upstream from the β-globin gene could contribute to
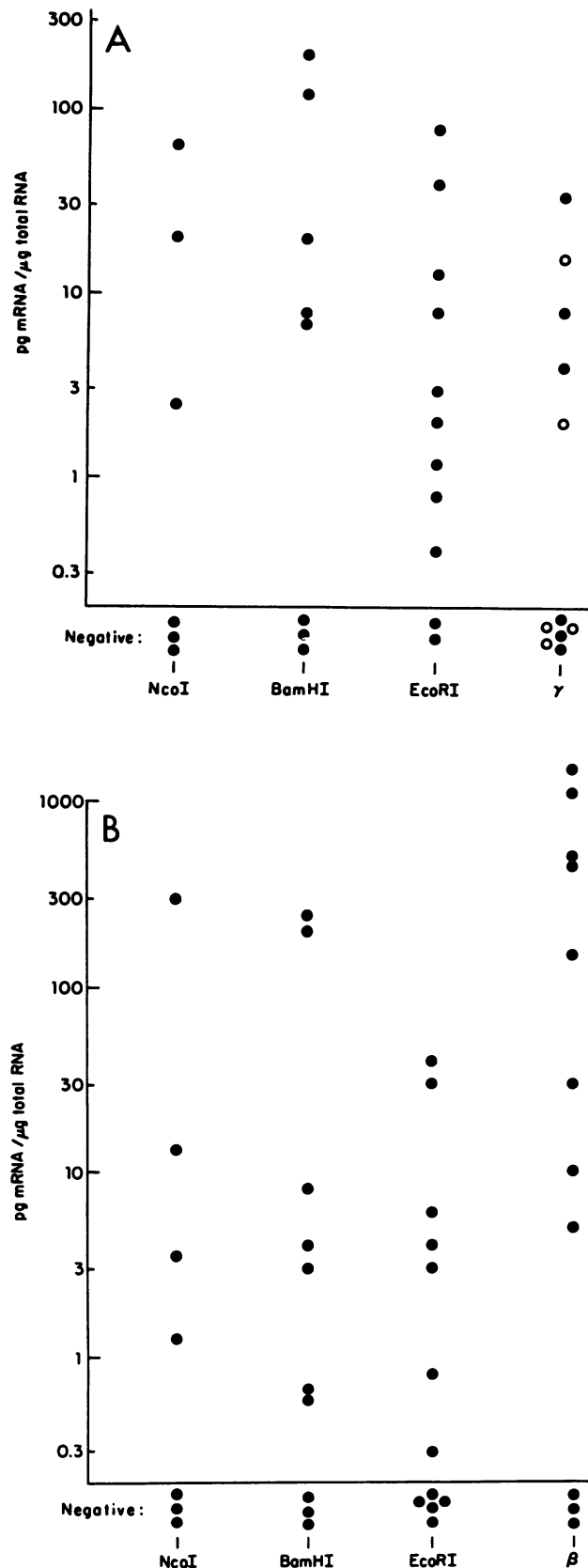
TABLE 1. Expression of 5'Gγ/3'β hybrid globin genes in erythroid tissues of embryonic, fetal, and adult transgenic mice[a]

Hybrid gene	Transgenic line	Expression level (pg of mRNA/μg of total RNA)			
		Embryonic blood	Fetal liver	Adult blood	Adult marrow
NcoI fusion	N4	20	60	13	13
	N12-22-1	0		0	
	N17	0		0	
	N33-3-1	65	150	300	75
	N33-3-8		7.5	3.5	1.25
	N39	2.5	2.5	1.2	0.5
	N53	0		0	
BamHI fusion	B5			8	
	B6-3	8		0.6	
	B6-5	200		200	100
	B10-3	2		0	
	B10-9	125	25	250	
	B12	0		0.6	
	B22-11	0		0	
	B24-9	20	40	3	0.6
	B25-3	0		0	
	B28-1	7.5	15	4	4
EcoRI fusion	E7	0.4		0	
	E10-1	0		0	
	E11-5	13		4	4
	E12	2		0	
	E14			0	
	E15-19	3	0.25	3	0.4
	E23-6	40	20	30	20
	E28	0.8		0.8	
	E30-31	8	2	6	4
	E34-1	0		0	
	E35-3	80	30	40	60
	E43	1.2		0.3	

[a] In transgenic lines designated by a single number (e.g., N4), adult blood and bone marrow samples were obtained from the founder animal, while embryonic blood and fetal liver samples were from F_1 progeny. In lines designated by a hyphenated number, all erythroid tissues analyzed were from F_1 to F_3 progeny. The detection limit in different experiments ranged from 0.1 to 0.3 pg of mRNA per μg of total RNA. mRNA levels were estimated by comparing hybridization signals obtained with transgenic mouse RNAs with the signals obtained with human K562 or human adult reticulocyte RNA (see Materials and Methods). The level of endogenous βh1-globin mRNA in 11.5-day-old mouse embryonic blood cells is approximately 200 pg/μg of total RNA (3), while the approximate levels of endogenous β-globin mRNA in mouse fetal liver, adult blood, and adult bone marrow are 1,000, 5,000, and 1,000 pg/μg of total RNA, respectively (4, 15).

the lack of expression at the embryonic stage. Our results are consistent with several studies with human K562 cells which indicate a positive role for upstream sequences in the expression of introduced γ-globin genes (1, 12; H. Lin, N. P. Anagnou, T. R. Rutherford, T. Shimada, and A. W. Nienhuis, J. Clin. Invest., in press).

Gγ-globin sequences between the initiation codon and the

FIG. 3. Comparison of levels of expression of 5'Gγ/3'β hybrid genes with fusion at the NcoI, BamHI, and EcoRI sites, in embryonic blood (A) and adult peripheral blood (B). Each dot represents a different transgenic line (data from Table 1). Also shown in panel A are data for a different transgenic mice carrying the Gγ-globin gene (●) or the Aγ-globin gene (○), (3, 24). Also shown in panel B are data for transgenic mice carrying an HindIII fragment including the entire β-globin gene (9; unpublished data). Dots below the abscissa represent mice in which there was no detectable expression of the transgene.

3' SphI site could be replaced with the corresponding β-globin sequences without any obvious effect on the levels of expression in mouse embryonic blood cells. Therefore, if any transcriptional regulatory elements reside in this region of the Gγ-globin gene, our data would suggest that they are not involved in the differential regulation of the Gγ-globin and β-globin genes in mouse embryonic erythroid cells.

Kollias et al. (13) reported the expression of a 5'Aγ/3'β hybrid globin gene (fused at the BamHI site) in embryonic, fetal, and adult erythroid cells of transgenic mice. In the single transgenic line for which the analysis was shown, the gene appeared to be expressed at a considerably higher level (at least 10-fold, if not 50-fold) in fetal liver than in embryonic blood, which is quite different than what we observed for any of the three Gγ/β hybrid genes. While this raises the possibility of functional differences between upstream Aγ-globin and Gγ-globin sequences, only a more extensive and quantitative analysis of the expression of Aγ/β hybrid globin genes would indicate whether the difference is significant. Based on the report of an enhancer active in K562 cells, downstream from the Aγ- but not the Gγ-globin gene (D. M. Bodine and T. J. Ley, submitted for publication), it would also be interesting to analyze the expression of a 5'β/3'Aγ hybrid globin gene in transgenic mice.

In contrast to the Gγ-globin gene, whose expression appears to depend primarily on upstream regulatory elements, the regulation of the adult β-globin gene seems not to depend to the same extent, if at all, on upstream sequences. Our results showed that β-globin sequences between −614 bp and the initiation codon were unable to cause the expression of a β/γ hybrid globin gene in mouse fetal or adult erythroid cells. We also found that mouse βmaj-globin upstream sequences failed to activate a heterologous reporter gene (mouse metallothionein I) in adult bone marrow or blood. Furthermore, Townes et al. (23) have shown that deletion of sequences upstream from −122 bp (relative to the cap site) of the β-globin gene had no apparent effect on the levels of expression in adult transgenic mice. In contrast, several experiments involving gene transfer into MEL cells have indicated a role for upstream β-globin sequences in erythroid cell specificity (22) as well as in the inducible expression of the β-globin gene during MEL cell differentiation (25), although others have not detected inducible expression of constructs containing only upstream β-globin sequences (5). The apparent discrepancy between our results and those obtained with MEL cell studies suggests that activation of the β-globin gene in the developing mouse may require cis-acting elements that are not necessary for expression of the transfected gene in MEL cells. In light of the above, it is interesting that the albumin gene, whose promoter region is sufficient to direct tissue-specific expression in transfection experiments (19), requires an additional enhancer element for appropriate expression in transgenic mice (20).

Intragenic β-globin sequences (from NcoI to BamHI or NcoI to EcoRI), together with upstream β-globin sequences, were also insufficient for expression in adult erythroid cells. Hybrid β/γ globin genes containing these sequences were expressed at low levels in only 1 of 13 and 1 of 12 transgenic lines, respectively. In these rare cases, it is possible that host DNA sequences at the site of integration exert a positive effect that overcomes a deficiency in the transgene.

Only hybrid globin genes that contained β-globin sequences downstream from the EcoRI site, including 3'-flanking sequences, were frequently expressed in fetal and adult erythroid cells. The expression of all three γ/β hybrid

genes contrasts sharply with the total lack of expression of γ-globin transgenes at these stages (3, 9, 13, 24). Therefore, β-globin sequences downstream from the EcoRI site seem to be essential for the expression of this gene in fetal and adult erythroid cells in vivo.

The three γ/β hybrid genes were generally expressed at much lower levels in adult blood cells than was an intact β-globin transgene (Fig. 3B); this could be due to a variety of factors. One possibility is that negative regulation of the Gγ-globin gene via an upstream element might depress the level of expression of γ/β hybrid genes in adult erythroid cells. Another is that while β-globin sequences 3' to the EcoRI site are sufficient for a low level of expression, the added effect of other intragenic or upstream β-globin sequences may be required for maximal expression.

Together with the recent demonstration of an enhancer in the 3'-flanking region of the chicken adult β-globin gene (7, 10), our results suggested that the human β-globin gene 3'-flanking region might contain an enhancer that is active in mouse fetal and adult erythroid cells. In another series of experiments, we confirmed the presence of an enhancer, situated approximately 600 to 900 bp downstream from the human β-globin polyadenylation site, which is able to activate an otherwise silent Gγ-globin gene in transgenic mouse fetal erythroid cells (M. Trudel and F. Costantini, Genes Dev., in press). The ability of the β-globin 3' enhancer to activate the Gγ-globin promoter can account for the expression of 5'γ/3'β hybrid globin genes at the fetal and adult stages.

Although the importance of human β-globin 3' sequences has been demonstrated in transgenic mice, these sequences presumably play a role in the stage-specific activation of the β-globin gene during human development. Interestingly, the naturally occurring human hemoglobin variant, Hb Kenya, contains a hybrid Aγ/β globin chain, which results from the deletion of sequences between the second exons of the Aγ-globin and β-globin genes (18). This hybrid gene is expressed in human adult blood cells at a level much lower than a normal β-globin gene but at higher levels than a normal Aγ-globin gene. It appears likely that the juxtaposition of β-globin 3' sequences close to the Aγ-globin promoter may be responsible for this effect. The Gγ-globin gene in cis to the Aγ/β fusion gene is also expressed in this condition, suggesting that the enhancing effect of β-globin 3' sequences may act over a distance of 7 kb.

ACKNOWLEDGMENTS

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