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Human γ- to β-Globin Gene Switching Using a Mini Construct in Transgenic Mice

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The developmental regulation of the human globin genes involves a key switch from fetal (γ-) to adult (β-) globin gene expression. It is possible to study the mechanism of this switch by expressing the human globin genes in transgenic mice. Previous work has shown that high-level expression of the human globin genes in transgenic mice requires the presence of the locus control region (LCR) upstream of the genes in the β-globin locus. High-level, correct developmental regulation of β-globin gene expression in transgenic mice has previously been accomplished only in 30- to 40-kb genomic constructs containing the LCR and multiple genes from the locus. This suggests that either competition for LCR sequences by other globin genes or the presence of intergenic sequences from the β-globin locus is required to silence the β-globin gene in embryonic life. The results presented here clearly show that the presence of the γ-globin gene (3.3 kb) alone is sufficient to down-regulate the β-globin gene in embryonic transgenic mice made with an LCR–γ-β-globin mini construct. The results also show that the γ-globin gene is down-regulated in adult mice from most transgenic lines made with LCR–γ-globin constructs not including the β-globin gene, i.e., that the γ-globin gene can be autonomously regulated. Evidence presented here suggests that a region 3′ of the γ-globin gene may be important for down-regulation in the adult. The 5′HS2γenβ construct described is a suitable model for further study of the mechanism of human γ- to β-globin gene switching in transgenic mice.

The human β-globin locus encompasses a region of 100 kb on chromosome 11 and includes the embryonic (ε-), fetal (γ-), and adult (β-) globin genes plus a regulatory region located 6 to 21 kb 5′ of the ε-globin gene (the locus control region [LCR]). Human γ- to β-globin gene switching is of interest as a model for developmental and tissue-specific gene regulation. It also has medical relevance, since increased fetal globin production in patients with adult hemoglobinopathies lessens the severity of these diseases (25). Although *cis*-acting elements and transcription factors involved in the erythroid cell-specific control of human globin gene expression are being identified (reviewed in reference 21), DNA sequences and factors responsible for developmental stage-specific regulation have not been found. Use of the transgenic mouse system is an appropriate way to study globin gene switching and is providing clues with respect to the regions required for this process. Constructs containing the human γ- or β-globin gene and the immediate flanking sequences are developmentally regulated in transgenic mouse assays. This shows that the *cis*-acting sequences necessary for this control are located near the genes themselves. In transgenic mice, the human γ-globin gene is expressed in the embryonic yolk sac (4, 17), and human β-globin is detected in the fetal liver and adult blood (7, 17, 26).

Although they are developmentally regulated, the human γ- and β-globin genes are expressed at very low levels in transgenic mice unless some or all of the LCR is included in the constructs. The LCR comprises a series of DNase I superhypersensitive sites (HS) restricted to erythroid cells (12, 28). A natural mutation deleting most of the LCR, but leaving the β-like globin genes intact, results in γδβ-thalassemia, suggesting that LCR elements normally act from a distance to greatly enhance the expression levels of these genes (9). This is confirmed in transgenic mouse experiments, in which position-independent and high-level expression of human globin gene constructs is achieved in the presence of the LCR (13). However, it appears that in some situations the LCR can override correct developmental control of the human globin genes in transgenic mice. For example, LCR–β-globin constructs are inappropriately expressed in the embryonic stage, instead of just the fetal and adult stages (2, 11). However, in transgenic mice with the LCR plus a chromosomally intact region containing the γ- and β-globin genes and their intervening sequence (~30 kb), the β-globin gene is correctly expressed at the fetal and adult stages. Enver et al. (11) and Behringer et al. (2) suggest that the LCR can interact with either the γ- or the β-globin gene at any given time, i.e., that these genes are competing for this upstream enhancer. The competition model is consistent with the observed deregulation of the LCR–β-globin construct; since there is no gene to compete with β-globin, it is expressed continuously. However, another possible interpretation of the results is that some intergenic sequence between the γ- and β-globin genes is required to silence the β-globin gene in the embryo. There is some controversy in the literature concerning correct developmental regulation of transgenic mouse constructs containing the LCR and the γ-globin gene alone. In some LCR–γ-globin constructs, the γ-globin gene is expressed at inappropriately high levels throughout development, although adult expression may be lower than embryonic/fetal expression (2, 10, 11). Dillon and Grosveld, however, have developed an LCR–γ-globin construct which is regulated normally; i.e., very low levels of γ-globin mRNA are found in adult transgenic mice, suggesting that the γ-globin gene can be autonomously regulated in the absence of the β-globin gene. This construct is different

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from the deregulated γ-globin gene constructs in that it contains a larger γ-globin gene fragment and a different LCR arrangement (8).

A number of questions remain unanswered concerning the mechanism of γ- to β-globin gene switching. To determine whether the presence of the γ-globin gene is sufficient to silence the β-globin gene during embryonic life, a mini construct (8 kb) containing the γ- and β-globin genes and a construct containing the β-globin gene alone were tested for correct developmental regulation in transgenic mice. The results of these experiments show that the γ-globin gene alone, without intergenic sequence from the β-globin locus, is sufficient to silence the β-globin gene. To attempt to more clearly define the sequences involved in developmental regulation of the γ-globin gene, two LCR–γ-globin constructs were made and tested in transgenic mice. The results show that the mechanism for down-regulation of the γ-globin gene in the adult is different from the mechanism for silencing the β-globin gene in the embryo, since the γ-globin gene is autonomously regulated in most transgenic lines. There may be an element involved in γ-globin gene silencing located within a 750-bp DNA fragment 3′ of the ^{A}γ-globin gene.

MATERIALS AND METHODS

5′HS2γ, 5′HS2γβ, and 5′HS2β constructs for transgenic mice. Each of the transgenic mouse constructs used in this work contains 5′HS2 from the human LCR. 5′HS2 is found within a 1.9-kb *Kpn*I-*Pvu*II fragment normally located about 11 kb 5′ of the ε-globin gene in the human β-globin locus. The human γ-globin gene is contained within a 3.3-kb *Hin*dIII fragment (−1350 to +1950). The γen constructs were made with a 4.1-kb γ-globin gene fragment, having an additional 750 bp of 3′ flanking sequence, ending at another *Hin*dIII site. The human β-globin gene used is a 4.5-kb *Apa*I-*Eco*RV fragment (−1250 to +3291) that includes the β-globin 3′ enhancer (1, 16, 27). This fragment contains the β^{s}-globin gene and was subcloned from the cosmid clone FC14 (6). These individual fragments were ligated into IBI30 or IBI31 plasmids to generate the 5′HS2γ, 5′HS2γen, 5′HS2γβ, 5′HS2γenβ, and 5′HS2β constructs. The inserts from these plasmids were gel purified prior to microinjection (15).

Generation of transgenic mice and DNA analysis. The 5′HS2γ and 5′HS2γen DNA constructs were microinjected into the male pronucleus of single-cell mouse embryos derived from B6C3F1 hybrid mice (15). For the 5′HS2γβ, 5′HS2γenβ, and 5′HS2β constructs, the fertilized mouse eggs were derived from the FVB/N strain. Transgenic mice (adults, embryos, or fetuses) were identified by using either Southern blots or the polymerase chain reaction for tail clip, ear clip, or placental DNAs. The integrity of the inserted DNA construct in each transgenic mouse line was confirmed with restriction endonuclease digestion and Southern blotting, using an enzyme cutting at diagnostic positions throughout the construct, ensuring that all components of the construct were intact. To avoid the quantitation problems ensuing from the use of F_0 mosaic animals, all of the 10.5-day embryos and 14.5-day fetuses used in the 5′HS2γ(en) and 5′HS2γ(en)β experiments are either F_1 or F_2 transgenic progeny. In the case of the 5′HS2γenβ4 and 5′HS2γβ3 lines, F_1 adults were not obtainable, and F_0 adult blood was used. The 5′HS2β mice analyzed are F_0 10.5-day embryos. In the gene counting experiments, fluorimetry was used to accurately determine the DNA concentrations of the transgenic samples. Hybridization on subsequent Southern blots was quantitated with a Molecular Dynamics PhosphoImager and ImageQuant software, and the transgene copy numbers were derived by comparison with a standard curve.

RNA analysis. Appropriate matings were done to isolate RNA (5) from the 10.5-day embryonic yolk sacs, 14.5-day fetal livers, and adult blood of the various transgenic lines. Relative levels of human γ- and β-globin versus mouse εy-, βh1- and β^{m}-globin mRNAs were quantitated by using the primer extension protocol previously described by Krakowsky et al. (18). The oligonucleotide probes used to detect globin mRNAs in these experiments are as follows: human γ (+86 to +105), 5′-TGCCCCACAGGCTTGTGATA-3′; human β (+78 to +95), 5′-CAGGGCAGTAACGGCAGA-3′; mouse εy (+58 to +75), 5′-TCCTCAGCAGTAAAGTTC-3′; mouse βh1 (+56 to +75), 5′-TCCTCAGCTGTGAAGT GAAC-3′; and mouse β^{m} (+66 to +85), 5′-AGCAGCCTTC TCAGCATCAG-3′. Primer extensions were quantitated by using the Molecular Dynamics PhosphoImager or scintillation counting of excised gel bands. The level of human globin mRNA was expressed as a percentage of the level of the endogenous mouse β-like globin mRNA(s) present at that time point (βh1 plus εy for embryonic; β^{m} for fetal and for adult). For comparison of these levels between transgenic lines, the values were divided by the copy number of the transgene per haploid genome for the transgenic line.

RESULTS

To further define the sequences necessary for developmental stage-specific expression of the human γ- and β-globin genes, and to test the competition model for silencing the human β-globin gene in the embryo, five constructs were made and analyzed in transgenic mice. Figure 1 shows these constructs (Fig. 1B) along with a map of the human β-globin locus from which they are derived (Fig. 1A). All of the constructs include 5′HS2, since it activates high-level expression of the human globin genes in transgenic mice (24). The 5′HS2γ and 5′HS2γen constructs include the human ^{A}γ-globin gene; 5′HS2γen contains 750 bp more sequence 3′ of the gene than does 5′HS2γ. This region (en) has been shown to exhibit enhancer activity in transient expression assays in tissue culture cells (3). The 5′HS2γβ and 5′HS2γenβ constructs additionally contain the human β-globin gene. These constructs differ from the human LCR–γ-β-globin constructs previously studied in transgenic mice (2, 11) in that the ~25 kb of sequence between the γ- and β-globin genes is excluded. The 5′HS2β construct contains the human β-globin gene alone. The expression levels of the human γ- and β-globin genes in the erythroid cells of the embryonic yolk sac, fetal liver, and adult blood of the transgenic mice were determined.

The β-globin gene is silenced in embryonic life in transgenic mice with an LCR–γ-β-globin mini construct. Previous transgenic mouse studies indicate that human LCR–β-globin constructs are inappropriately expressed at the embryonic stage of development. In genomic LCR–^{A}γ-^{G}γ-ψβ-δ-β-globin constructs (30 to 40 kb), however, the β-globin gene is expressed only at the fetal and adult stages (2, 11). To determine whether the presence of the γ-globin gene alone is sufficient to silence the β-globin gene in embryonic transgenic mice, five transgenic lines with the 5′HS2γ(en)β mini construct were studied. Figure 2A depicts a primer extension analysis of RNA from the yolk sacs of mouse embryos from each of these transgenic lines. Probes specific for human γ- and β-globin mRNA (hγ and hβ) and for the mouse

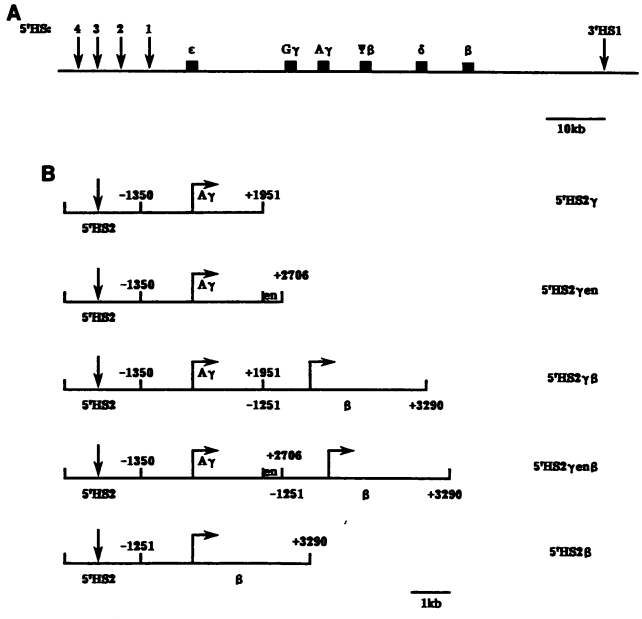

FIG. 1. Human β-globin locus and constructs used to make transgenic mice. (A) The ~100-kb human β-globin locus, including the β-like globin genes and the HS located 5′ and 3′ of the genes. The HS are identified by arrows, and the relative positions of the genes are shown. The ε-globin gene is normally expressed predominantly in the embryonic yolk sac, the ^{G}γ- and ^{A}γ-globin genes are expressed in the fetal liver, and the δ- and β-globin genes are expressed in the adult bone marrow. The 5′HS2 site, located ~11 kb upstream of the ε-globin gene, was included in the γ- and γ-β-globin gene transgenic mouse constructs depicted in panel B. (B) Transgenic mouse constructs. Each construct includes a 1.9-kb *Kpn*I-*Pvu*II fragment containing 5′HS2. The 5′HS2γ and 5′HS2γen constructs contain 3.3- and 4.1-kb γ-globin gene *Hin*dIII fragments, respectively. en is a 750-bp region 3′ of the γ-globin gene previously shown to have enhancer activity in transient assays. The 5′HS2γβ and 5′HS2γenβ constructs additionally contain a 4.5-kb *Apa*I-*Eco*RV β-globin gene fragment. The 5′HS2β construct contains the β-globin gene fragment alone. The start sites and direction for transcription of the γ- and β-globin genes are indicated.

βh1-, εy-, and β^m-globin mRNAs (mβh1, mε, and $m\beta^m$) were included in this experiment. The sum of mouse βh1 and εy expression represents the total mouse endogenous globin RNA at the embryonic time point.

Lane 1 of Fig. 2A is a nontransgenic mouse negative control in which the mouse ε/βh1 product of 75 bases is visible, and there are no products corresponding to the human γ- and β-globin probes at 105 and 95 bases. As expected, the human γ-globin gene is expressed at high levels in the embryonic yolk sac in all five transgenic mouse lines (top bands, lanes 2 to 6). The levels of γ-globin mRNA are comparable to endogenous mε/mβh1 levels (bottom bands, lanes 2 to 6). As indicated in the quantitative results in Table 1, γ-globin expression per transgene copy ranges from ~9 to 50% of endogenous levels in the five lines tested, averaging 23%. The human β-globin gene, however, is expressed at very low or undetectable levels in the 10.5-day embryos from either the 5′HS2γβ or 5′HS2γenβ lines. This result is in contrast to those for LCR–β-globin constructs, which are expressed at high levels at the embryonic time point (2, 11). The results show that the presence of the γ-globin gene alone (3.3 kb) can silence the β-globin gene in embryonic life. The low level of human β-globin in the 5′HS2γβ1 and 5′HS2γβ2 samples is probably due to contamination with adult blood from the maternal transgenic mouse, since these are also the only samples which contain small amounts of mouse adult β^m mRNA (hβ and $m\beta^m$; lanes 2 and 3).

At the fetal time point, γ-globin expression levels remain high compared with endogenous mouse β^m-globin mRNA levels (Fig. 2B, lanes 2 to 4; Table 1). Although detectable levels of γ-globin expression are not observed in the fetal livers of transgenic mice containing human γ-globin constructs without LCR sequences (4, 17), in humans the γ-globin gene is normally expressed in the fetal liver. Human β-globin mRNA is also detected in the 14.5-day liver samples of the three transgenic lines tested. In general, there are higher levels of human γ- than β-globin mRNA at this time point (Table 1).

In adult blood, the ratio of γ- to β-globin mRNA shifts (Fig. 2C), so that less γ-globin mRNA and greater quantities

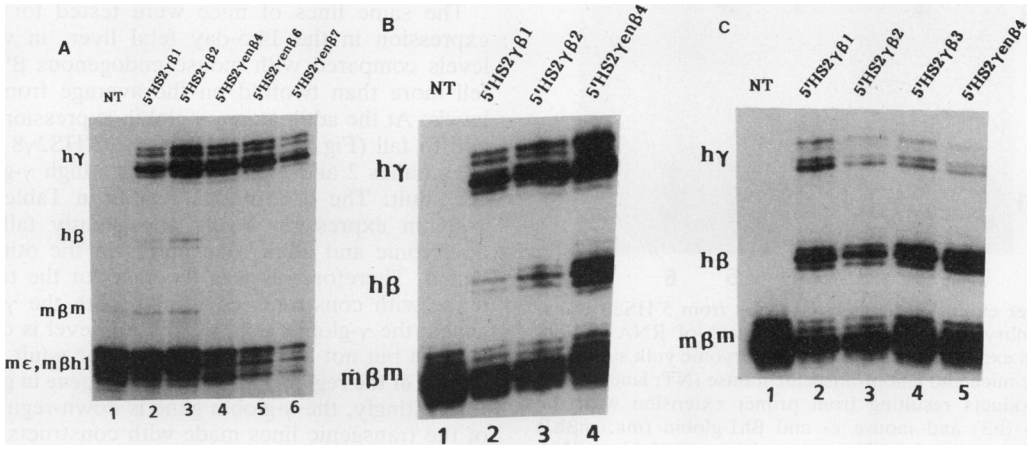

FIG. 2. Primer extension analysis of RNA from 5′HS2γβ and 5′HS2γenβ transgenic mouse tissues. The developmental time points and tissue sources for the RNAs used in the primer extensions are 10.5-day embryonic yolk sac (A), 14.5-day fetal liver (B), and adult blood (C). The transgenic mouse line tested is indicated above each lane; NT is a nontransgenic negative control sample (lane 1). The lengths of the primer extension products from correctly initiated globin mRNAs are as follows: human γ (hγ), 105 bases; human β (hβ), 95 bases; mouse β^m ($m\beta^m$), 85 bases; and mouse εy and βh1 (mε, mβh1), 75 bases.

TABLE 1. Expression per transgene copy of γ- and β-globin in 5'HS2γβ and 5'HS2γenβ transgenic mice

Transgene	Line	% β-Globin expression/transgene copy[a]			% γ-Globin expression/transgene copy[a]			Copy no.[b]
		10.5 day	14.5 day	Adult	10.5 day	14.5 day	Adult	
5'HS2γβ	1	0.3	1.8	14.4	8.8	13.7	8.4	3
	2	0.9	6.4	9.1	20.9	21.3	2.4	3
	3	ND	ND	2.7	ND	ND	0.5	10
5'HS2γenβ	4	0.0	5.0	3.7	10.0	9.5	0.4	10
	6	0.0	ND	ND	49.6	ND	ND	3
	7	0.0	ND	ND	27.8	ND	ND	6

[a] Human γ- or β-globin expression is represented as a percentage of the level of endogenous β-like globin RNA expressed at the indicated time point: βh1 plus εy for 10.5-day embryos; β^m for 14.5-day fetuses and adults. This percentage has been corrected to represent expression level per transgene copy. ND, data not available.

[b] Copies of the transgene per haploid genome.

of human β-globin mRNA are observed in adult blood in the three transgenic lines tested in all cases (lanes 2, 3, and 5; Table 1). Table 1 shows that the level of γ-globin expression decreases between the embryonic and adult stages in the 5'HS2γβ2 (9-fold) and 5'HS2γenβ4 (25-fold) lines. In the 5'HS2γβ1 line, however, γ-globin expression levels are about the same at the adult and embryonic time points (~8%). Therefore, the γ-globin gene is transcriptionally down-regulated in two of three 5'HS2γ(en)β transgenic mouse lines.

Since the LCR–β-globin constructs previously studied in transgenic mice had either all four 5'HS or 5'HS1 and 5'HS2, we needed to confirm that a 5'HS2β construct is indeed expressed inappropriately at the embryonic time point. The expression level of human β-globin at the 10.5-day time point was determined in six individual 5'HS2β F_0 embryos (Fig. 3, lanes 1 to 6). Four of the transgenic embryos showed high

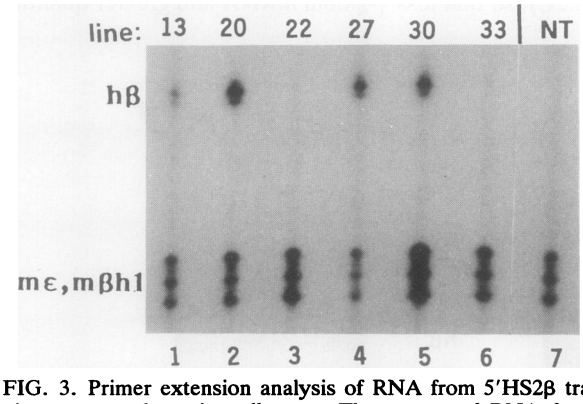

FIG. 3. Primer extension analysis of RNA from 5'HS2β transgenic mouse embryonic yolk sacs. The source of RNA for the primer extension experiments is 10.5-day embryonic yolk sacs from six F_0 transgenic mice and a nontransgenic mouse (NT; lane 7). The sizes of the products resulting from primer extension with the human β-globin (hβ) and mouse ε- and βh1-globin (mε, mβh1) probes are indicated at the left. The percent human β-globin mRNA per gene copy for the six embryos tested are (in the order shown) 25.2, 69.0, 0.0, 38.9, 23.3, and 3%. Copy numbers per haploid genome ranged from one to three and are approximate because they were established from placental DNA samples, and it is possible that these F_0 embryos are mosaic. Abbreviations are as for Fig. 2.

levels (23 to 69%) of human β-globin mRNA per gene copy compared with the total endogenous levels of mouse ε plus βh1 (lanes 1, 2, 4, and 5). Two of the transgenic embryos exhibited low or undetectable levels (0 and 3%) of human β-globin mRNA (lanes 3 and 6). It is possible that these embryos were mosaic and/or that human β-globin expression was subject to negative insertional position effects. It is clear, however, that the average level of human β-globin expression at the 10.5-day time point in the 5'HS2β mice is much higher than that in the 5'HS2γ(en)β mice and that the β-globin gene is deregulated in 5'HS2β embryos.

The γ-globin gene is generally down-regulated in adult transgenic mice with an LCR–γ-globin construct not containing the β-globin gene. To probe the mechanism by which the γ-globin gene is silenced in the adult, a series of transgenic lines made with either the 5'HS2γ or slightly larger 5'HS2γen construct was studied. Although some LCR–γ-globin constructs are expressed at high levels in the adult (2, 10, 11), work by Dillon and Grosveld suggests that the γ-globin gene can be silenced in adult transgenic mice without the presence of the β-globin gene (8). Primer extension experiments were carried out to determine the levels of γ-globin expression at the embryonic, fetal, and adult time points for five 5'HS2γ and four 5'HS2γen transgenic mouse lines.

Figure 4A shows the results for the embryonic yolk sac RNAs; it is evident that considerable levels of γ-globin mRNA are expressed in the 5'HS2γ (lanes 2 to 6) and 5'HS2γen (lanes 7 to 10) lines. Interestingly, increased expression levels were not evident (Table 2) with the constructs containing the additional 750-bp 3' flanking sequence (en), previously shown to increase expression levels of a chloramphenicol acetyltransferase reporter gene in transient assays. Rather, γ-globin mRNA levels for transgenic lines made with either the 5'HS2γ or 5'HS2γen construct range from ~2 to 15% at this time point. Another interesting point evident from Fig. 4A and Table 2 is that the transgenic lines made with constructs having the γ-globin gene alone (5'HS2γ and 5'HS2γen) appear to express less γ-globin mRNA at 10.5 days (mean, 5.5%) than do those made with the γ- and β-globin genes (5'HS2γβ and 5'HS2γenβ; Table 1; mean, 23%). It is possible that the β-globin gene is up-regulating the γ-globin gene and that this effect might be important in vivo.

The same lines of mice were tested for γ-globin gene expression in the 14.5-day fetal liver, in which γ-globin levels compared with mouse endogenous β^m-globin levels fell more than twofold on the average from the 10.5-day levels. At the adult stage, γ-globin expression levels continued to fall (Fig. 4C) except in the 5'HS2γ8 and 5'HS2γ73 lines (lanes 2 and 3), which express high γ-globin levels in the adult. The quantitative results in Table 2 reveal that γ-globin expression levels consistently fall between the embryonic and adult time points in the other seven lines tested. Therefore, as was the case for the transgenic lines made with constructs containing both the γ- and β-globin genes, the γ-globin gene expression level is down-regulated in most but not all 5'HS2γ lines in the adult.

Role of the region 3' of the γ-globin gene in gene regulation. Interestingly, the γ-globin gene is down-regulated in all five of the transgenic lines made with constructs which contain the 750-bp 3' γ-globin flanking sequence (en). Three of the seven lines made with constructs not having this sequence were not down-regulated (5'HS2γβ1, 5'HS2γβ8, and 5'HS2γ73). The results are consistent with the idea that this DNA fragment not only does not act as an enhancer in

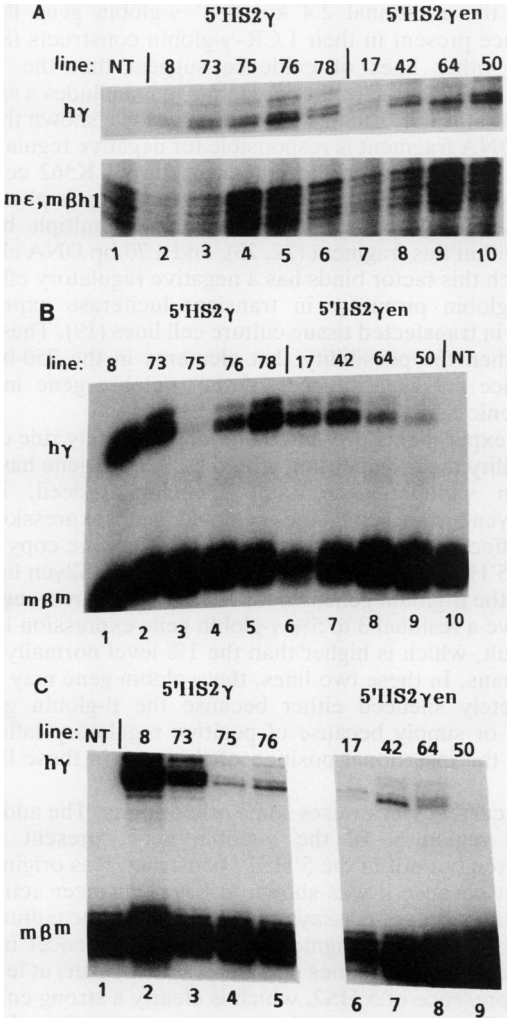

FIG. 4. Primer extension analysis of RNA from 5'HS2γ and 5'HS2γen transgenic mouse tissues. The sources of RNA for the primer extension experiments are 10.5-day embryonic yolk sac (A), 14.5-day fetal liver (B), and adult blood (C). The transgenic mouse line from which the RNA was extracted is indicated above each lane. NT is a nontransgenic negative control sample (lane 1). The probes included in the primer extension are the same as in Fig. 2, and the products resulting from correctly initiated mRNAs are indicated at the left (hγ, human γ-globin; mε and mβh1, mouse embryonic globins; and $m\beta^m$, mouse fetal and adult globin). The primer extension experiment for the adult blood sample from the 5'HS2γ78 line is not shown, but the quantitative data for this line are included in Table 2.

transgenic mice but might actually contain an element capable of silencing the γ-globin gene in adult animals. The fold difference between the level of γ-globin expression at the embryonic and the adult time points for each of the 5'HS2γ and 5'HS2γen lines tested is shown in Table 2. On the average, γ-globin gene expression fell less than 3-fold for the 5'HS2γ lines but 20-fold for the 5'HS2γen lines. Therefore, the γ-globin gene is down-regulated in more lines and to a greater extent in 5'HS2γen than in 5'HS2γ transgenic mice. Primer extension analysis of several additional 5'HS2γen transgenic lines was carried out to determine their γ-globin expression levels at the adult time point (quantitative results are shown in Table 3). On average, the 5 5'HS2γ lines

TABLE 2. γ-Globin expression in individual 5'HS2γ and 5'HS2γen lines through development

Transgene	Line	% γ-Globin expression/ transgene copy[a]			Fold decrease in % γ-globin expression[b]	Copy no.[c]
		10.5 day	14.5 day	Adult		
5'HS2γ	8	2.2	3.8	2.0	1.1	13
	73	3.7	3.1	5.2	0.7	10
	75	14.0	2.8	2.7	5.2	1
	76	4.1	1.1	0.8	5.1	8
	78	1.9	3.0	0.7	2.7	14
5'HS2γen	17	0.5	1.0	0.1	5.0	38
	42	2.1	1.4	0.3	7.0	14
	64	14.9	5.0	3.1	4.8	1
	50	6.3	0.4	0.1	63.0	6

[a] See Table 1, footnote *a*.
[b] Fold decrease in percent γ-globin expression per transgene copy between the 10.5-day and adult time points.
[c] See Table 1, footnote *b*.

expressed 2.3% γ-globin in the adult, while the 12 5'HS2γen lines had somewhat lower adult levels (1%). Taken together, these results suggest that the 750-bp γ-globin gene 3' flanking fragment could contain a DNA element(s) responsible for down-regulating γ-globin gene expression in the adult.

DISCUSSION

To better understand the mechanisms of human γ- to β-globin gene switching, a series of DNA constructs including the γ-globin gene, both the γ- and β-globin genes, or the β-globin gene were introduced into transgenic mice. Previous studies show that LCR–β-globin constructs are not developmentally regulated in transgenic mice. However, the β-globin gene is expressed with adult stage specificity in genomic (~30-kb) LCR–γ-ψβ-δ-β-globin constructs. These results led Behringer et al. (2) and Enver et al. (11) to propose a model of β-globin gene silencing in the embryo based on competition between the γ- and β-globin gene promoters for interactions with upstream LCR sequences. The theory states that in the absence of the γ-globin gene, the β-globin promoter freely interacts with the LCR at the embryonic time point, resulting in inappropriate β-globin expression. Another possible interpretation of their data is that intergenic sequences between the γ- and β-globin genes are required to silence the β-globin gene in the embryo. If the competition model is correct, the β-globin gene should be developmentally regulated in transgenic mice with a smaller construct containing only the γ- and β-globin genes. The

TABLE 3. Adult γ-globin expression in additional 5'HS2γen transgenic mouse lines

Line	% γ-Globin expression/ transgene copy[a]	Copy no.[b]
41	0.7	9
51	1.0	2
53	0.1	13
65	0.5	2
81	0.1	2
83	0.6	8
91	0.4	2
95	4.7	3

[a,b] See Table 1, footnotes *a* and *b*.

work presented here showed that the insertion of a 3.3-kb fragment containing the γ-globin gene in a 5′HS2β construct is sufficient to turn the β-globin gene off in the embryo. This result is consistent with the competition model for silencing of the β-globin gene in the embryo and rules out the possibility that other sequences between the γ- and β-globin genes are required for this effect. Following the submission of this report, Hanscombe et al. (14) also demonstrated correct expression of the human γ- and β-globin genes in transgenic mice, using an abbreviated construct with all four 5′HS preceding the two genes. Our studies indicate that 5′HS2 alone is sufficient for developmental regulation of the genes. There still remains the possibility that some element within the γ-globin gene fragment silences the β-globin gene through a mechanism other than competition between the gene promoters for interactions with the LCR. Deletion analysis of the 5′HS2γenβ mini switching construct should provide a good test for the competition model.

Down-regulation of the γ-globin gene in the adult seems to involve a mechanism other than competition with the β-globin gene. In fact, the γ-globin gene was down-regulated (3- to 60-fold) in seven of the nine transgenic lines tested which were made with LCR constructs containing the γ-globin gene and no other genes. If the γ-globin gene can be down-regulated to this extent, it can be regulated autonomously; i.e., the presence of the β-globin gene is not required to silence the gene in the adult. This interpretation of the results is somewhat in contrast to those of Behringer et al. (2) and Enver et al. (11). They have reported inappropriately high levels of γ-globin expression in adult transgenic mice with LCR–γ-globin constructs containing the same γ-globin gene fragment used in 5′HS2γ (2, 10, 11). However, only one or two transgenic animals were examined at the adult time point by each of the two groups. For example, Behringer et al. (2) reported that two lines of LCR–γ-globin transgenic mice expressed an average of 16% γ-globin mRNA in adult blood, representing a sixfold drop (from 100% in 11-day embryos). This result led the authors to conclude that temporal specificity of γ-globin gene expression was lost. Our interpretation of the results is closer to that of Dillon and Grosveld, who have developed an LCR–γ-globin construct which is down-regulated 30- to 250-fold during development in several lines of transgenic mice (the low copy number of these lines may influence the greater decrease in expression observed by these authors) (8). This construct contains an additional 2.4 kb of 3′ γ-globin flanking sequence that was not present in the initially studied constructs. The 5′HS2γen construct contains 750 bp of this additional sequence.

The results presented here suggest the possibility that a silencer element within this 750-bp sequence (en) is responsible for negatively regulating the γ-globin gene at the adult stage. The γ-globin gene was down-regulated in the adult in all but 3 of the 12 transgenic lines containing LCR–γ-globin or LCR–γ-β-globin construct which were tested at three time points. These three adults were all from 5′HS2γ or 5′HS2γβ lines, not 5′HS2γen or 5′HS2γenβ lines, which contain constructs including the additional 750-bp (en) 3′ γ-globin gene flanking region. Indeed, the average fold decrease in γ-globin levels from the embryonic to adult time points is several times higher for the five 5′HS2γen(β) (21-fold) than for the seven 5′HS2γ(β) (4-fold) lines. This evidence suggests that a silencer sequence(s) may be located in the +1951 to +2706 (en) region of the γ-globin gene. Dillon and Grosveld had previously speculated that a silencer may lie within the additional 2.4 kb of 3′ γ-globin gene flanking sequence present in their LCR–γ-globin constructs (8).

Two other lines of evidence suggest that the 750-bp *Hin*dIII fragment 3′ of the ^{A}γ-globin gene includes a silencer element(s). First, Lumelsky and Forget have shown that this same DNA fragment is responsible for negative regulation of γ-globin gene transcription when erythroid K562 cells are induced with phorbol esters to a megakaryocytic phenotype (20). Second, a novel nuclear factor has multiple binding sites within this fragment (22, 23), and a 70-bp DNA element to which this factor binds has a negative regulatory effect on the γ-globin promoter in transient luciferase expression assays in transfected tissue culture cell lines (19). These data strengthen the possibility that elements in the 750-bp (en) sequence act as a silencer of the γ-globin gene in adult transgenic mice.

The experiments presented do not completely rule out the possibility that competition with the β-globin gene has some role in γ-globin gene adult silencing. Indeed, in the 5′HS2γenβ transgenic line, γ-globin gene expression was very effectively silenced to 0.4% per transgene copy in the adult (5′HS2γenβ4; Table 1). Two of the 5′HS2γen lines, in which the β-globin gene is not present, were down-regulated but have a residual 3 to 5% γ-globin gene expression level in the adult, which is higher than the 1% level normally found in humans. In these two lines, the γ-globin gene may not be completely silenced either because the β-globin gene is absent or simply because of positive regulatory influences due to the insertional position of the gene in these lines of mice.

The current work raises some other points. The additional 750-bp region 3′ of the γ-globin gene, present in the 5′HS2γen but not in the 5′HS2γ construct, was originally of interest because it was shown to have enhancer activity in transient expression assays in transfected tissue culture cells (3). However, this fragment may act as a silencer in adult transgenic mice and does not act as an enhancer, at least not in the presence of 5′HS2, which is clearly a strong enhancer itself. The percent γ-globin gene expression for the 5′HS2γen lines is not greater than for the 5′HS2γ lines at the embryonic time point (Table 2). Although this fragment contains binding sites for the positive erythroid regulator GATA-1 (19, 23), its positive role in regulation is still unclear.

Another interesting finding of this work is that the presence of the β-globin gene appears to enhance embryonic expression of the γ-globin gene in transgenic mice. The 5′HS2γ(en) lines had an average γ-globin expression level of ~6% in the embryo, while the average for the 5′HS2γ(en)β lines was fourfold higher. This enhancement of γ-globin gene expression in 5′HS2γ(en)β transgenic mice may be fortuitous due to the close proximity of the β-globin gene to the γ-globin gene in this construct, but it may be that elements in the β-globin gene or β-globin 3′ enhancer play a role in regulating the γ-globin gene in vivo. The β-globin expression levels per transgene copy at the adult time point are lower than previously reported by others for a 5′HS2β construct. It is unclear whether this is due to chance or to the presence of the γ-globin gene in close proximity to the β-globin gene in our constructs.

Although the γ- and β-globin genes are developmentally regulated in LCR–γ-β-globin constructs, their time frame for switching appears to be different from the original γ- or β-globin constructs tested without LCR sequences. In transgenic mice made with LCR–γ-β-globin or β-globin constructs, the human β-globin gene is expressed in the fetal

liver as well as in the adult animal (2, 7, 11, 17, 26), following a pattern similar to that for the endogenous mouse β^m-globin gene. Without LCR sequences, the fetal human γ-globin gene is expressed mainly in the embryonic yolk sac (4, 17), at the same time that the endogenous mouse embryonic εy and βh1 genes are expressed. When LCR sequences are included, as in the 5'HS2γ(en)β constructs, the γ-globin gene is also expressed in the fetal liver, and at a higher level than is the human β-globin gene. This is actually closer to the human situation, where the γ-globin gene is expressed in the fetal liver and down-regulated in the adult.

The 5'HS2γenβ transgenic mouse is suitable as a simple switching model, since the β-globin gene is clearly silenced in the embryo and the construct includes the 3' sequences which may silence the γ-globin gene in the adult. The 5'HS2γenβ construct should provide a good starting point for mutational and deletional analysis of the γ-globin and β-globin genes, to pinpoint the sequences near the genes which are necessary for correct developmental switching. The 5'HS2γenβ transgenic mice may also provide a useful γ- to β-globin switching model for testing drugs which elevate γ-globin levels in adult animals and studying the mechanism by which they do so.

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