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Regulated Expression of the Human β Globin Gene in Transgenic Mice Requires an Upstream Globin or Nonglobin Promoter

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Transgenic mice have been used extensively to study elements governing the erythroid-specific developmental switch from human fetal γ to human adult β globin. Previous work demonstrated that a small construct composed of hypersensitive site 2 (HS2) of the locus control region (LCR) linked to the γ and β globin genes (HS2-γ-β) is sufficient for correct tissue and temporal expression of these genes, whereas HS2-β alone is inappropriately expressed in the embryo. Two models, which are not mutually exclusive, have been proposed to explain these results and those of other constructs in transgenic mice. One model emphasizes the conserved polarity in the globin locus and suggests a distance effect whereby the β globin gene must be removed from the LCR/HS2 to prevent an early and incorrect activation of this gene in the embryonic compartment. A second hypothesis proposes a competition between the γ and β globin gene promoters for interaction with the LCR/HS2. The active γ globin gene promoter positioned between the LCR/HS2 and the β globin gene thereby interacts with the HS2 elements early in erythroid development and is expressed until a change in putative stage-specific nuclear factors makes an interaction with the adult β globin gene more favorable. In an effort to test the competition model, a construct has been prepared in which a small deletion was produced in the promoter region of the γ globin gene while in the context of the HS2-γ-β plasmid. Analysis of this construct in transgenic mice reveals a constitutive unregulated expression of the human β globin gene during erythroid development. To determine if this competition effect is specific for globin genes, a heterologous reporter gene has been substituted for the γ globin gene in the construct HS2-γ-β. In this case, the β globin gene exhibits correct developmental expression. This data is consistent with a model in which transcription from a promoter upstream of the β globin gene in some manner protects this adult gene from activation by the LCR/HS2 during early development.

INTRODUCTION

The human β globin locus is located on chromosome 11 and includes the gene set 5′-ε-Gγ-Aγ-δ-β-3′. These genes are developmentally expressed in a temporal manner that corresponds to their gene order in the locus. The embryonic gene is expressed in the yolk sac, the γ globin genes are active in the fetal liver, and the δ and β globin genes are expressed in adult bone marrow (reviewed in Collins and Weissmann, 1984). This locus is flanked by super-hypersensitive sites that are erythroid-specific and present at all developmental stages (Tuan *et al.*, 1985; Forrester *et al.*, 1986). A cluster of four hypersensitive sites, spaced over approximately 20 kilobase (kb), are present 6 kb upstream of the ε globin gene. A single hypersensitive site has also been observed 19 kb downstream from the β globin gene. These sites have been termed the locus control region (LCR) and are believed to promote a chromatin conformation that

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results in the erythroid-specific activation of the globin locus.

Studies in transgenic mice using globin miniconstructs in which surrounding sequences have been deleted while the hypersensitive sites are maintained have shed light on the function of these sites (Collis *et al.*, 1990). Recent work has indicated that these elements confer erythroid-specific, position-independent, high-level expression to globin genes or heterologous genes (Grosveld *et al.*, 1987; Blom van Assendelft *et al.*, 1989). This is in contrast to earlier studies in transgenic mice in which expression of globin gene constructs was significantly influenced by the site of integration of the transgene. Even when correct tissue and developmental expression were observed, the levels of mRNA produced were very low compared to the mouse endogenous genes (Costantini *et al.*, 1985; Townes *et al.*, 1985; Chada *et al.*, 1986).

Various combinations of the four hypersensitive sites 5′ of the locus have been tested for their ability to confer this high-level expression in a tissue-specific manner. Although the effect is maximal with all four sites, hypersensitive sites 2 (HS2) and 3 have been found to be critical for the activity (Collis *et al.*, 1990). Site 2 alone confers erythroid-specific expression of globin genes at levels averaging 40% of the mouse endogenous globins (Ryan *et al.*, 1989). Further deletions in HS2 indicated that there are multiple elements required for the activity but that a fragment as small as 1.9 kb can confer the maximal effect for this site (Caterina *et al.*, 1991).

The constructs testing the tissue-specific expression of the intact or dissected LCR in many instances utilized the β globin gene. It was noted both with the entire LCR (Behringer *et al.*, 1990; Enver *et al.*, 1990) and with HS2 alone (Lloyd *et al.*, 1992) that the developmental control of the adult β globin gene is lost. The gene is only expressed in erythroid tissues, but it is prematurely activated in the embryonic period and continues to be expressed throughout development. In contrast, transgenic mice produced with constructs of LCR-γ or HS2-γ express the γ globin gene in the embryonic yolk sac but exhibit downregulation of the gene in adult blood (Enver *et al.*, 1989, 1990; Dillon and Grosveld, 1991; Lloyd *et al.*, 1992). These results suggest that the γ globin gene can be autonomously regulated, whereas control of the β globin gene is overridden when it is linked directly to the LCR/HS2. When larger constructs composed of the entire globin locus plus the LCR are expressed in transgenic mice, correct temporal regulation of both the γ and the β globin genes is observed (Behringer *et al.*, 1990; Enver *et al.*, 1990). This has led to the proposal of a promoter competition between the γ and β globin genes for interaction with the LCR. Thus, in the absence of γ globin gene competition, the β globin gene is constitutively expressed. Alternatively, intergenic sequences present in the large constructs could be supplying necessary sequences that contribute to the appropriate expression of the globin genes. An additional model for the regulation of the γ and β globin genes suggests that the polarity of the locus and the relative distances of these genes from the hypersensitive sites may be important in determining which gene has a more favorable interaction with the LCR (Hanscombe *et al.*, 1991). This model was developed on the basis of the results obtained with constructs such as LCR-γ-β, where correct temporal expression of both genes is obtained whereas the reversal of the gene order in LCR-β-γ results in correct γ globin expression but aberrant early expression of the β globin gene.

Recently, we have demonstrated that a construct containing only HS2 of the LCR followed by the γ and β globin genes (HS2-γ-β) is capable of directing the correct expression of these genes in transgenic mice (Lloyd *et al.*, 1992). Other investigators have also obtained similar results with a construct containing HS2 (Morley *et al.*, 1992). By manipulating this "mini-switching" construct, we have tested what sequence elements within the HS2-γ-β construct might be responsible for one aspect of globin gene switching, that is, the lack of transcription of the β globin gene in the embryonic yolk sac. A construct deleting a small fragment containing basal promoter elements of the γ globin gene was designed to determine whether the presence of the γ globin gene promoter is necessary to silence the β globin gene early in development. Furthermore, we have substituted a heterologous gene for the γ globin gene in the HS2-γ-β construct to analyze the effect of an active nonglobin gene on the developmental expression of the human β globin gene in transgenic mice.

MATERIALS AND METHODS

Preparation of Plasmid DNA

HS2-$\gamma\Delta$p-β was produced by eliminating a region containing the promoter of the human γ globin gene and reconstructing the plasmid analogous to the HS2-γ-β plasmid used previously (Lloyd *et al.*, 1992). Briefly, the HS2 region is a 1.9-kb *Kpn* I-*Pvu* II fragment located about 11 kb 5′ of the ϵ globin gene in the human β globin locus. The γ globin gene is contained within a *Hin*dIII fragment (−1350 to +1950); however, two *Nco* I fragments encompassing the region between −140 and +284 have been deleted. 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 (Behringer *et al.*, 1987; Kollias *et al.*, 1987; Trudel *et al.*, 1987) and was subcloned from the cosmid clone FC14 (Collins *et al.*, 1984). The individual fragments were ligated into IBI30 or 31 plasmids to generate the HS2-$\gamma\Delta$p-β construct. The insert from this plasmid was gel purified and subjected to CsCl gradient centrifugation before microinjection (Hogan *et al.*, 1986).

HS2-TKCAT-β was generated using the minimal thymidine kinase (TK) promoter from −200 to +53 (McKnight *et al.*, 1981). Briefly, the HS2-β construct previously described (Lloyd *et al.*, 1992) was cleaved at the *Sal* I site between HS2 and the β globin gene (−1250 to +3291) and filled in. A TKCAT (1.8 kb) fragment was removed with *Sac* I from pUC18 and ligated into HS2-β. The resulting plasmid was purified using Qiagen plasmid kits (Qiagen, Chatsworth, CA), and the insert was isolated for microinjection.

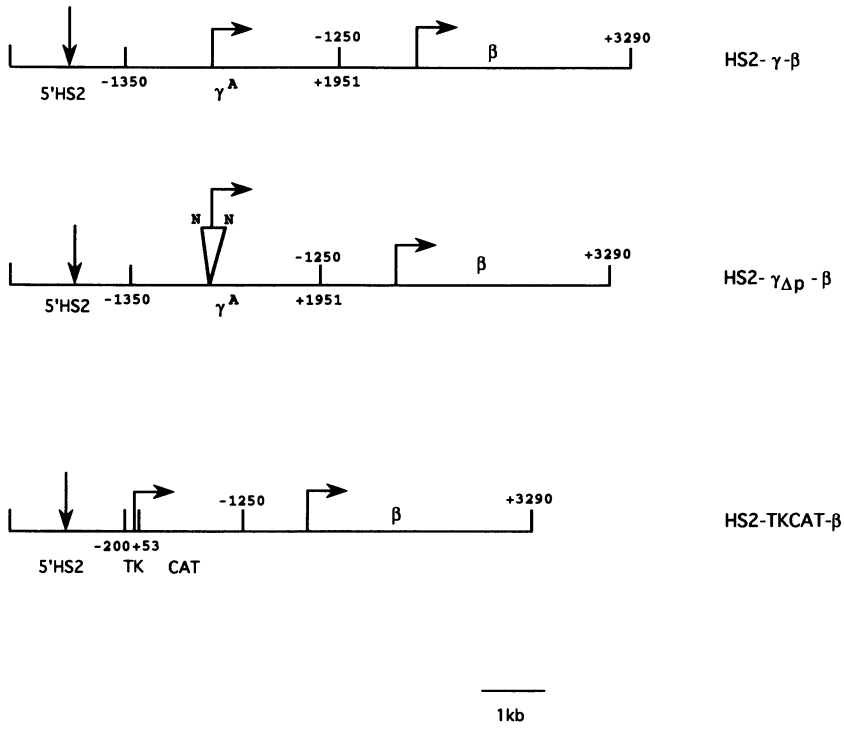

Figure 1. Schematic diagram of the mini-switching construct and the derived constructs. HS2-γ-β is the switching construct previously reported (Lloyd *et al.*, 1992). HS2-γΔp-β is the promoterless γ globin gene construct in which a 424-bp region flanked by *Nco* I sites has been removed. HS2-TKCAT-β contains the CAT reporter gene driven by the TK promoter substituted for the γ globin gene. In each case, HS2 is marked by a down arrow within the 1.9-kb *Kpn* I-*Pvu* II fragment, the nucleotide positions of the start and end sites of the genes are indicated, and the transcription start sites are designated with arrows.

Generation and Analysis of Transgenic Mice

Transgenic mice were produced, and the integrity of the inserted DNA was confirmed by Southern blot analysis using probes for the HS2 fragment as well as the specific genes for each construct. This ensured that all components of the construct were intact. In experiments to determine gene copy number, fluorimetry was used to accurately quantitate the DNA concentrations of the transgenic samples. Transgene copy numbers were derived by comparing to a standard curve. RNA was prepared from various tissue sources and analyzed by primer extension as previously described (Lloyd *et al.*, 1992). A Molecular Dynamics (Sunnyvale, CA) PhosphoImager and ImageQuant software were used to quantitate the hybridization signals.

Chloramphenicol Acetyltransferase (CAT) Assays

Assays for CAT activity were performed as described by Gorman *et al.*, (1982) from cellular extracts of yolk sacs, fetal liver, or blood prepared by freeze-thaw lysis. The reactions were incubated for 3 h at 37°C before extraction with ethylacetate and separation of the products by thin layer chromatography. Protein concentrations of each CAT extract were quantitated using the Bio-Rad Protein Assay (Richmond, CA) solution.

RESULTS

Earlier work from our laboratory (Lloyd *et al.*, 1992) has demonstrated that a mini-switching construct (HS2-γ-β) containing only HS2 followed by the γ and β globin genes is capable of correct tissue and developmentally specific expression of the human globin genes in transgenic mice. This is in contrast to the results we and others (Lloyd *et al.*, 1992; Morley *et al.*, 1992) have observed with HS2 and the β globin gene alone (HS2-β). In this latter case, the gene is expressed constitutively during development. To test whether this is because of the absence of a competitive promoter or the placement of the β globin gene in the direct proximity of HS2, we have prepared the plasmid constructs shown in Figure 1.

We first examined the effect of ablating transcription from the γ globin gene promoter, while maintaining the body of the γ globin gene and the approximate distance between HS2 and the β globin gene. The parent construct that was altered is HS2-γ-β. This construct was previously shown to direct the correct expression of the γ globin gene in the embryonic yolk sac and the β globin gene in adult blood (Lloyd *et al.*, 1992). HS2-γΔp-β was produced by deleting a 424-base pair (bp) fragment containing the promotor elements of the γ globin gene. After microinjection into the pronuclei of fertilized mouse eggs, F0 embryos containing the HS2-γΔp-β construct were collected at 10.5 d for embryonic yolk sac, at 14.5 d for fetal liver, or allowed to develop to term to establish transgenic mouse lines.

Assays for the expression of the transgene were carried out by primer extension analyses. RNA was prepared from the yolk sacs, fetal liver, or adult blood of transgenic mice. Levels of the human β globin transgene expression were compared to the levels of endogenous mouse εy and βh1 mRNA in the yolk sac samples and mouse β^m mRNA in the fetal liver and blood samples, and calculated as a function of gene copy number. Figure 2 shows the β globin primer extension results with RNA isolated from F0 yolk sacs and fetal livers. All five

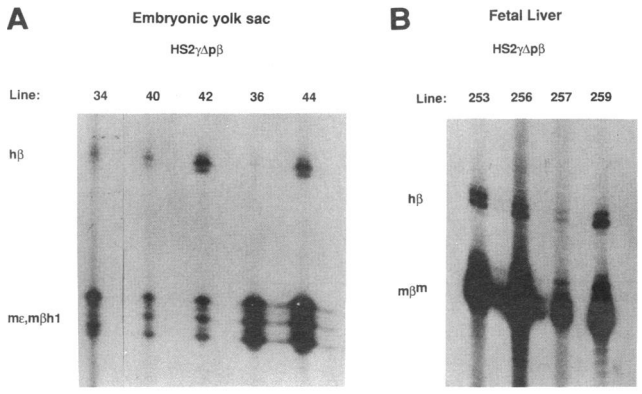

Figure 2. Primer extension analysis of RNA prepared from transgenic yolk sacs and fetal livers after microinjection of the HS2-γΔp-β construct. The yolk sac samples in A were collected at 10.5 d, and the liver samples in B were isolated at 14.5 d after microinjection. The line numbers indicated the F0 embryos and fetuses obtained from these direct dissections. hβ indicates the human β globin product. mε, mβh1, and $m\beta^m$ mark the position of the mouse endogenous primer extension products.

of the 10.5-d yolk sac samples (Figure 2A) demonstrate expression of the adult β globin gene in the embryonic compartment. The copy number corrected levels of expression relative to the mouse endogenous globin mRNAs are presented in Table 1 and ranged from 4% (lines 36 and 44) to 60% (line 42). In transgenic lines with the HS2-γ-β construct, β globin gene expression in the embryonic compartment is always <1% (Lloyd *et al.*, 1992). The fact that the human β globin gene is expressed in all five of the yolk sacs suggests that the β globin gene will be constitutively expressed in mice carrying this promoterless γ globin gene construct. The expression of the human β globin gene was therefore followed into the fetal liver while the primary site of erythropoiesis shifted during development. Primer extensions were performed on RNA from four F0 14.5-d fetal livers. All four samples exhibit expression of the transgene. These results are shown in Figure 2B. The levels of transgene expression and number of copies of the construct for each of these injections is included in Table 1.

Two transgenic lines were established with the HS2-γΔp-β construct. The mice were bred to obtain RNA from 10.5-d yolk sacs, 14.5-d fetal livers, and adult blood. Although we had observed expression of the human β gene at 10.5 d from direct dissections, we also wished to demonstrate the constitutive developmental expression of the human gene within a line of mice. Primer extension analyses are shown in Figure 3 for these two lines. Both lines exhibit constitutive expression of the human β globin gene at approximately the same level throughout development. Although the level of

Table 1. Quantitation of human β globin expression relative to mouse β-like globins

Transgene	Line	Sample	% β globin gene expression[a]	Transgene copy number	Corrected β globin expression[b] (%)
HS2-γΔp-β	34	10.5-d yolk sac	13	5	5
	36	10.5-d yolk sac	2	1	4
	40	10.5-d yolk sac	29	3	19
	42	10.5-d yolk sac	60	2	60
	44	10.5-d yolk sac	10	5	4
	253	14.5-d fetal liver	3	3	2
	256	14.5-d fetal liver	1	2	1
	257	14.5-d fetal liver	3	1	6
	259	14.5-d fetal liver	1	1	2
	3	10.5-d yolk sac	11	2	11
		14.5-d fetal liver	9		9
		Adult blood	9		9
	7	10.5-d yolk sac	22	16	3
		14.5-d fetal liver	33		4
		Adult blood	11		1
HS2-TKCAT-β	8	10.5-d yolk sac	0	4	0
		14.5-d fetal liver	58		29
		Adult blood	93		46
	22	10.5-d yolk sac	0	4	0
		14.5-d fetal liver	13		6
		Adult blood	18		9
	26	10.5-d yolk sac	0	22	0
		14.5-d fetal liver	34		3
		Adult blood	150		14

[a] Expression is presented as a percentage of the endogenous mouse globin, either βh1 and εy for yolk sac or β^m for fetal liver and adult blood samples.

[b] Values from column 4 were corrected by dividing by the copy number and multiplying by 2 for the two mouse β globin genes per haploid genome.

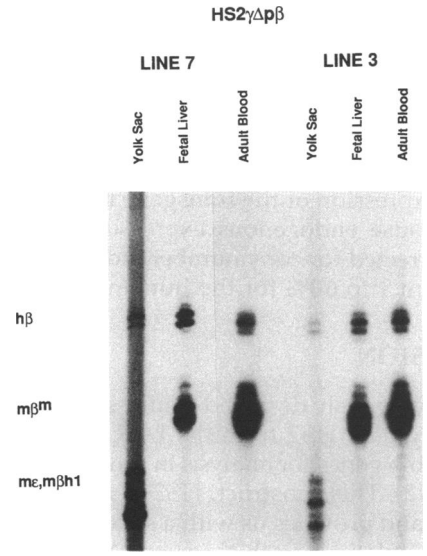

Figure 3. Primer extension analysis of RNA from tissue samples of HS2-γΔp-β transgenic mouse lines. The source of mRNA for the primer extension experiments are 10.5-d embryonic yolk sac, 14.5-d fetal liver, and adult blood. The transgenic mice lines are indicated at the top of each composite. The products resulting from correctly initiated mRNAs are indicated at the left of the figure: hβ, human β globin; $m\beta^m$, mouse fetal and adult globin; mε and mβh1, the mouse embryonic globins.

expression in the yolk sac of line 3 in this figure appears decreased compared to that seen in the adult, the endogenous mouse globin signal is also less. Thus, the relative expression as quantitated by phosphoimager analysis and presented in Table 1 is approximately equivalent at all time points. The human β globin gene, therefore, is clearly expressed at all developmental stages in these lines, in accordance with the results from the direct dissections.

Although these results indicate that the promoter of the γ globin gene is necessary for the correct developmental expression of the adult β globin gene, an interesting question is whether this apparent requirement for transcription is globin-gene specific. For example, is a specific interaction between the HS2 and a globin promoter necessary to protect the β globin gene from early expression or is the requirement only for transcription from any active promoter. To approach this aspect, we produced a similar construct containing a heterologous gene in place of the γ globin gene. In this plasmid, called HS2-TKCAT-β, expression of the CAT reporter gene (Gorman *et al.*, 1982) is driven by a minimal 253-bp thymidine kinase promoter (McKnight *et al.*, 1981). The schematic diagram of HS2-TKCAT-β is included in Figure 1. After injection of this insert into the pronuclei of fertilized mouse eggs, three transgenic mouse lines were established. The lines were bred, and 10.5-d embryonic yolk sacs, 14.5-d fetal livers, and adult blood samples were obtained for analysis. Total RNA was isolated and assayed by primer extension for the expression of the human β globin gene. The results of these analyses are shown in Figure 4. With this construct, the regulation of the β globin gene is maintained by the replacement of the γ globin gene with another actively transcribed gene, in this instance, TKCAT. In all the lines, the β globin gene is not expressed in the 10.5-d yolk sac, but the mRNA is evident in the 14.5-d fetal liver and adult blood samples. Similar results were also obtained with two F0 embryos and an F0 fetus obtained from direct dissections of litters from a separate microinjection with the HS2-TKCAT-β construct.

In contrast with this developmentally regulated globin gene expression, Figure 5 illustrates the CAT activity present in aliquots of the same samples as those used in the primer extension analyses. The CAT gene is expressed constitutively in all samples. In these assays, the relative levels between different types of tissues are not directly comparable because the extracts contain differing proportions of erythroid-specific proteins (e.g., liver versus blood). Furthermore, the assays for CAT and human β globin gene expression differ, one measuring enzyme activity and the other mRNA levels, precluding any calculation of relative activities. Attempts at direct comparisons of these expression levels have not been successful because of inherent problems with the erythroid developmental system. For example, primer extension analysis to compare CAT and globin mRNA is difficult because of the short half-life of CAT mRNA (1 h versus 60 h for globin). This analysis is

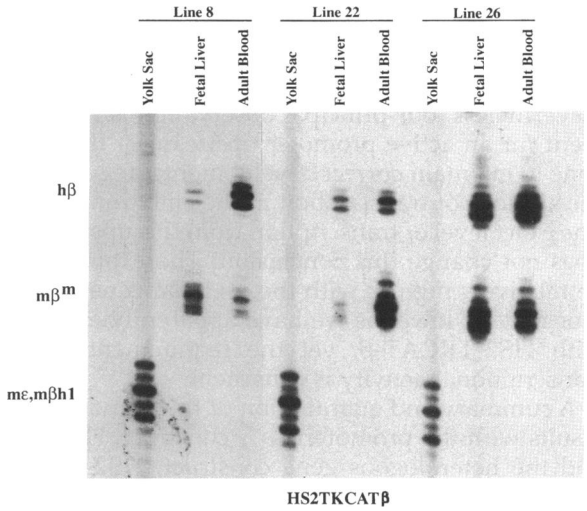

Figure 4. Primer extension analysis of samples from transgenic mice produced with the construct HS2-TKCAT-β. Line numbers are used to identify the transgenic mice that were obtained. The timepoints chosen for the dissections were 10.5 d for the yolk sacs and 14.5 d for fetal livers. These individual tissues were split into two samples, one for the primer extension analysis and the other for the determination of CAT activity. The primer extension products are identified as hβ for human β globin, $m\beta^m$ for mouse β major, and mε and mβh1 for the mouse embryonic globins.

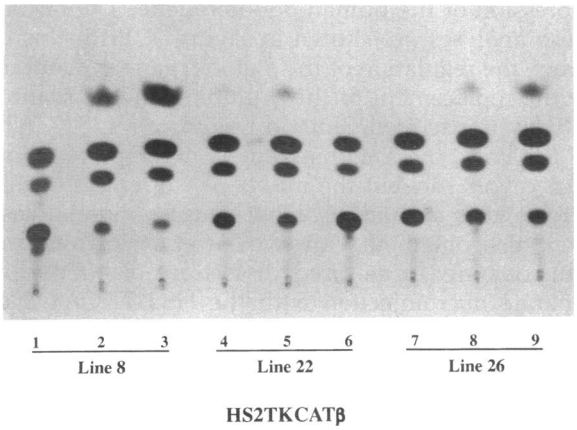

Figure 5. Analysis of the CAT activity in tissue samples from three lines carrying the transgene HS2-TKCAT-β. Cell extracts were prepared from 10.5-d yolk sacs, 14.5-d fetal livers, and adult blood for each line. These were the same samples used in the primer extension analysis shown in Figure 4. A CAT assay was performed, and the products of this assay, unreacted chloramphenicol and its acetylated forms, were separated by thin layer chromatography. Lanes 1, 4, and 7, yolk sac; lanes 2, 5, and 8, fetal liver; lanes 3, 6, and 9, adult blood. The amount of each cell extract assayed for this figure are as follows: lane 1, 3, μg; lane 2, 22 μg; lane 3, 56 μg; lane 4, 6 μg; lane 5, 21 μg; lane 6, 46 μg; lane 7, 12 μg; lane 8, 31 μg; lane 9, 16 μg.

further complicated by the small yields of mRNA obtained from embryonic yolk sacs and the fact that red blood cells have no nucleus to sustain transcription of the CAT gene. Additionally, Western blot analysis of the relative protein levels for CAT and human and mouse globin was investigated. Unfortunately, commercial antibodies are not available that distinguish mouse from human β globin, nor is there an antibody available that specifically recognizes mouse ϵ globin. Nevertheless, our principal observation is the requirement for an active promoter upstream of the β globin gene to maintain correct developmental regulation, i.e., the absence of transcription in the embryonic yolk sac. The exact level of transcription from this upstream gene does not change this conclusion. The expression may be relatively high as with the γ globin gene in HS2-γ-β or may be lower as we have apparently experienced with HS2-TKCAT-β, yet the requirement for some transcriptional activity is consistent.

A summary and quantitation of the primer extension results with the promoterless γ construct, HS2-$\gamma\Delta$p-β, and the heterologous gene construct, HS2-TKCAT-β, are presented in Table 1. Transgene copy numbers were determined from the placentas of the embryonic and fetal F0 mice and on tail DNA from mice of the established lines. For the HS2-$\gamma\Delta$p-β founder animals used directly from injections, one cannot rule out the possibility of mosaicism in the placenta as compared to other tissues. The level of expression per copy number for these samples is therefore approximate. Although copy number dependence of expression has been observed by some groups (Grosveld *et al.*, 1987; Blom van Assendelft *et al.*, 1989; Fraser *et al.*, 1990; Philipsen *et al.*, 1990), we and others have not necessarily noted this relationship in our data (Ryan *et al.*, 1989; Shih *et al.*, 1990; Lloyd *et al.*, 1992; Morley *et al.*, 1992). Rather, our results indicate consistent and significant erythroid-specific expression of the transgene to levels equivalent to the mouse endogenous expression in many cases. When corrected for copy number, however, these levels range from 1 to 60% for the human β globin gene.

DISCUSSION

We had previously designed a mini-switching construct containing only HS2 from the LCR with the human γ and β globin genes for analysis in transgenic mice (Lloyd *et al.*, 1992). This construct, HS2-γ-β, proved to switch properly and provides us with a small construct for further manipulations in this system. As a control for this experiment, we had also produced a construct, HS2-β, which lacked the γ globin gene. In mice carrying this latter construct, the human β globin gene is inappropriately expressed in the embryo. Two models have been proposed to explain these results. The models themselves are not mutually exclusive but highlight different potential control points for globin gene regulation. One model emphasizes the conserved polarity in the globin locus and suggests that the relative distance of the γ and β globin genes from the LCR/HS2 plays a role in preventing an early and incorrect activation of the β globin gene in the embryonic compartment (Hanscombe *et al.*, 1991). Perhaps, therefore, the position of the β globin gene at a distance from the LCR/HS2 is critical to its regulation. A second hypothesis proposes a competition between the γ and β globin gene promoters for interaction with the LCR/HS2 (Behringer *et al.*, 1987). The active γ globin gene promoter, positioned between the LCR/HS2 and the β globin gene, would thereby protect the β globin gene from the effects of the LCR/HS2 until the environment of the bone marrow makes this latter interaction more favorable. In an effort to test these two models, two globin constructs were prepared.

First, a small deletion was produced in the promoter region of the γ globin gene while in the context of the HS2-γ-β plasmid. The results from the analysis of this construct in transgenic mice reveals constitutive unregulated expression of the human β globin gene during early embryonic development. These results are seen both in the analysis of yolk sacs dissected 10.5 d after microinjection and in the yolk sacs of 10.5-d embryos from two transgenic lines. This data is therefore consistent with a model requiring an active promoter upstream of the β globin gene to protect this adult gene from activation by the LCR/HS2 during early development. In addition, the data suggests that the distance between HS2 and the β globin gene is not of primary importance. The distance is not changed very much

from HS2-γ-β, yet the β globin gene is constitutively expressed in HS2-γΔp-β.

One question raised by these experiments is the required specificity of the upstream promoter. Hanscombe *et al.* (1991) report correct regulation of the human β globin gene in a transgenic mouse with an LCR construct containing the α globin gene positioned 5′ of the β globin gene. It is possible that the LCR/HS2 must interact specifically with a globin promoter, either from the γ gene or the α gene, to open the chromatin domain for regulated expression. It is known, however, that the LCR can confer tissue specificity on heterologous genes in mouse erythroleukemia cells (Talbot *et al.*, 1989). Therefore, perhaps transcription of any gene would be sufficient to suppress inappropriate early expression of the human β globin gene. A construct that substituted a nonglobin gene in the region upstream of the β gene was prepared to address this question. In the construct HS2-TKCAT-β, the reporter is driven by the TK promoter because this basal promoter was expected to give constitutive expression during development. Indeed, our results demonstrate that this gene is active at all times during erythroid differentiation, whereas expression of the human β globin gene is suppressed in the embryonic yolk sac and activated in the later developmental stages. This suggests that an important factor in maintaining regulated β globin gene expression appears to be the transcription of a gene between the HS2 and the β globin gene. This gene need not be a member of the globin family, nor is it necessary that the gene be downregulated in the adult compartment.

This latter aspect is of interest, because it implies that a reciprocal relationship in the expression of the globin genes may not be a driving factor in their regulation. Studies with the γ globin gene have already suggested that this gene is capable of autonomous regulation. Downregulation of γ globin gene expression in adult transgenic mice is observed with the constructs LCR-γ and HS2-γ (Enver *et al.*, 1989, 1990; Dillon and Grosveld, 1991; Lloyd *et al.*, 1992) in the obvious absence of a corresponding upregulation of a β globin gene. Additionally, the experiments of Hanscombe *et al.* (1991) provided evidence that regulated expression of the β globin gene may not require a reciprocal gene downregulation. In their study, the α globin gene is constitutively expressed, whereas the β globin gene is correctly regulated in transgenic mice bearing the insert μLCR-α-β. This report extends that analysis by the introduction of a heterologous gene in the place of the α globin gene. With the HS2-TKCAT-β construct, suppression of the β globin gene in the embryonic period and activation in the adult compartment is accompanied by a constitutive developmental expression of the nonglobin CAT gene.

One additional comparison relating to distance from the HS2 elements is from the work of Hanscombe *et al.* (1991) concerning the result from an inversion of the α and β genes relative to the μLCR, i.e., μLCR-$\tilde{\alpha}$-$\tilde{\beta}$. In this instance, the human β globin gene was expressed in the embryonic yolk sac. This could be explained on the basis of tandem integrations of multiple copies of the transgene, resulting in an array that positions the β globin gene promoter closer to the LCR elements (μLCR-$\tilde{\alpha}$-$\tilde{\beta}$-μLCR . . .) than the α globin gene promoter. Such an arrangement behaves as an LCR-β-α construct where the proximity of the β globin gene to the LCR stimulates developmentally constitutive expression. This was not the case with our HS2-TKCAT-β construct however, presumably because even in a tandem array the small TK promoter is always closer to the HS2 elements than the β globin gene promoter, and thus it protects the adult globin gene.

The levels of expression of the β globin gene in these constructs are slightly less than that normally seen with the use of a single hypersensitive site. Ryan *et al.* (1989) reported an average copy-number-corrected expression level for human β globin as 40% of the mouse endogenous globin when using the HS2 site alone. The range they observed was 6–84% per copy with a 5.8-kb fragment containing HS2 and 13–63% per copy with a smaller 1.9-kb fragment identical to that used in our constructs. If only the established lines are considered, our range is 3–46% with an average human β globin gene expression of 16% of the mouse endogenous β globin gene. If F0 embryos are also included in this analysis, the range of transgene expression per copy number expands to 1–60% of endogenous expression with an average value of 13%. Because these are not bred lines however, the copy number determined may represent a mosaicism. This would be a serious concern in interpretation if no expression was observed, because mosaicism could result in very low levels of transgene RNA. However, because expression of the human β globin gene is present in all F0 samples from the HS2-γΔp-β construct and is confirmed in the lines of mice, these are significant positive findings for the constitutive nature of this expression. It is not clear why our average expression levels are reduced compared to that seen by Ryan *et al.* (1991) with a similar hypersensitive site construct. Nevertheless, these levels are still easily detectable and are similar to those reported previously for the parent HS2-γ-β mini-switching construct (Lloyd *et al.*, 1992) that maintains the correct developmental pattern of expression.

We have noted that the range of expression observed in this work and in studies by other investigators (Ryan *et al.*, 1989; Shih *et al.*, 1990; Lloyd *et al.*, 1992; Morley *et al.*, 1992) indicates copy number independent expression of the transgene with a number of constructs. This is in contrast with results reported by other groups utilizing either HS2 or the entire LCR (Grosveld *et al.*, 1987; Blom van Assendelft *et al.*, 1989; Fraser *et al.*, 1990; Philipsen *et al.*, 1990). One explanation for copy

number independence in such experiments may be the titration of limited transcription factors required for expression in the high copy number animals. It is not clear, however, if this fully explains the discrepancy between the groups. Nevertheless, our results demonstrate high level, erythroid-specific expression of the human globin transgenes in a developmentally specific manner using the mini-switching construct.

A model for adult β globin gene regulation consistent with the results presented here might therefore incorporate two levels of control. One level is the suppression of the β globin gene during embryonic life. Our principle observation is the apparent requirement for embryonic transcription of a gene upstream of the β globin gene in the context of the LCR/HS2. The constitutive expression of the β globin gene observed with the promoterless γ globin gene construct suggests the requirement for an active globin promoter upstream of the β globin gene. Interaction between the HS2 element and this promoter could act to protect the adult β globin gene from activation during early development. It is possible that in this context, however, all that is required is the formation of a transcription complex, perhaps even a noncompetent complex, rather than the actual transcription of a gene. It would therefore be interesting to mutate a sequence for a basic transcription factor in the γ globin gene promoter and assay for the effect on human β globin gene regulation in our mini-switching construct. The restoration of regulated β globin gene expression in the construct that substituted a heterologous gene for the upstream γ globin gene implies that a globin-specific interaction with the HS2 is not required. Rather, transcription of a heterologous, nonglobin gene, or perhaps just the interaction of its active promoter with the HS2, can result in the suppression of the β globin gene during embryonic life. To overcome this early shielding of the β globin gene from the HS2, a second layer of control could be invoked in the model suggesting the presence of a stage-specific factor that upregulates the β globin gene, possibly even in the face of continuous expression of the heterologous upstream gene. This raises an additional issue of stage-specific binding factors whose levels may vary during development. Recently, a stage-specific factor for the γ globin gene was reported (Jane *et al.*, 1992). The sequence element that this putative factor binds is located within the region deleted in the promoterless γ gene construct described here. The precedence exists therefore for a factor that could upregulate the human β globin gene in a similar manner. Experiments are currently underway to test these hypotheses.

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