The human β -globin locus activation region alters the developmental fate of a human fetal globin gene in transgenic mice

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ABSTRACT We linked a 3.3-kilobase fragment containing the entire $\Lambda\gamma$ -globin gene together with 1.3 kilobases of 5' flanking and 0.37 kilobase of 3' flanking DNA to a 2.5-kilobase fragment containing four of the developmentally stable hypersensitive sites normally located in the 5' region of the human β -globin locus. This construct was injected into fertilized mouse eggs, and its expression was analyzed in the primitive and definitive erythroid cells, as well as the brain of 14-day embryos. All six transgenic individuals that contained intact copies of the construct expressed the transgene in an erythroid-specific fashion. Expression was observed in both primitive and definitive erythroid cells. This is in marked contrast to previous transgenic mice experiments using the same $\Lambda\gamma$ -globin gene fragment in isolation, where expression was restricted to primitive erythroid cells. Our results show that the region containing the developmentally stable globin locus hypersensitive sites changes the developmental stage specificity of a human fetal globin gene in transgenic mice. These observations imply that sequences additional to those used here are involved in the developmental control of fetal globin gene expression *in vivo*. The ability to express fetal globin in adult erythroid cells allows one to consider using fetal globin genes for gene therapy of sickle cell disease.

The developing human progressively switches from embryonic ($\zeta_2\epsilon_2$, $\alpha_2\epsilon_2$, and $\zeta_2\gamma_2$) to fetal ($\alpha_2\gamma_2$) and finally adult ($\alpha_2\delta_2$ and $\alpha_2\beta_2$) hemoglobin production (reviewed in ref. 1). These developmental changes are largely attributable to the differential expression of the genes of the β -globin locus. This locus contains five functional genes spanning 60 kilobases (kb), arranged in the order 5' ϵ -G γ - $\Lambda\gamma$ - δ - β 3'. Switches in the expression of these genes during ontogeny are accompanied by changes in the site of erythropoiesis. Thus, ϵ expression is associated with yolk sac erythropoiesis, G γ and $\Lambda\gamma$ expression with fetal liver erythropoiesis, and δ and β expression with adult bone marrow erythropoiesis.

Structural analyses of the β -globin cluster have revealed both developmentally regulated and developmentally stable chromatin features. DNase I-hypersensitive sites located in the promoters of the individual globin genes are present only when the specific genes are being expressed (2). In addition to the developmentally regulated hypersensitive sites local to the individual globin genes, there is a second class of hypersensitive sites that are rather more remote. These are located 6–18 kb upstream of the ϵ -globin and 20 kb downstream of the β -globin genes (3, 4). They are erythroid specific and developmentally stable and are present regardless of which globin genes are active.

Reverse genetics experiments in transgenic mice (5–10) using relatively small globin gene-containing fragments have shown that sequences important for the developmental stage specificity of globin gene expression are located in the

immediate vicinity of the genes themselves. For example, the human β -globin gene is expressed only in the definitive and not the primitive erythroid cells of transgenic mice (5, 6, 8–10). Similarly, when human fetal globin genes are introduced into transgenic mice, they are expressed only in primitive and not in definitive erythroid cells (7, 8). A fusion gene containing the 5' portion of a human fetal globin gene and the 3' portion of the human adult β -globin gene is expressed throughout mouse erythroid development (8). However, in all these experiments, the expression of the introduced gene was found to be only a fraction of that of the endogenous cellular globin genes and was dependent on the site of integration into the host genome (11). Studies in somatic cell hybrids (12), transgenic mice (13), and cell lines (14) have shown that the region containing the developmentally stable hypersensitive sites located 5' to the human ϵ -globin gene is important for high-level position-independent β -globin gene expression. This region termed the locus activation region (LAR) has also been shown to confer high-level erythroid-specific expression on heterologous erythroid (α -globin) as well as lymphoid (Thy-1) genes (15, 16), demonstrating that the effect is not promoter specific. Thus to achieve ordered developmental regulation of the β -globin locus, the individual β -like globin genes must escape the effect of the LAR at specific stages of development. The goal of the experiments reported here was to explore this possibility. A 3.3-kb $\Lambda\gamma$ -globin gene-containing fragment is developmentally regulated (as a mouse embryonic gene) when introduced into transgenic mice (7, 8), implying that this fragment contains sequences important in developmental regulation. We asked if these same sequences would be sufficient to maintain developmental control when linked to the LAR.

MATERIALS AND METHODS

pBS μ LAR $\Lambda\gamma$ Transgenic Mice. pBS μ LAR $\Lambda\gamma$ contains four developmentally stable hypersensitive sites from the 5' region of the β -globin locus within a 2.5-kb fragment (14) linked to a 3.3-kb fragment containing a marked copy of the $\Lambda\gamma$ -globin gene (17) in pBluescript SK(–) (Stratagene). The fragments containing the hypersensitive sites are as follows: *Hind*III 13,769 to *Nco*I 13,062; *Bgl*II 9218 to *Hind*III 8486*; *Hind*III 5172* to *Bal*I 4608*; *Sph*I 1702* to *Pst*I 1182. The $\Lambda\gamma$ -globin gene fragment is *Hind*III 38,084 to *Hind*III 41,382. Restriction sites destroyed during cloning are indicated by an asterisk. Numbers indicate the positions of the restriction sites (GenBank locus HUMBB accession no. J00179) in the β -globin locus. The μ LAR- $\Lambda\gamma$ insert was released from the vector by cleaving at the *Not*I and *Sal*I sites in the pBluescript SK(–) polylinker and was used to make transgenic mice (18).

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Abbreviation: LAR, locus activation region.

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Nucleic Acid Analysis. DNA was prepared from frozen brain and fetal liver samples as described (19). Carcasses, yolk sac, and placental material were ground under liquid nitrogen prior to DNA extraction performed as described (20). Nucleic acid concentrations were determined by UV spectroscopy or fluorimetry, and nucleic acid integrity was assessed by agarose gel electrophoresis. Slot blot analysis of DNA was performed as described by Kafatos *et al.* (21) with uniformly labeled probes for the -18 and -15 hypersensitive regions and exon 3 of the human γ -globin gene (7). Southern blot analysis was performed as previously described (20). RNA was prepared as described in ref. 19. One-tenth microgram of RNA was used in each RNase protection assay, which was performed as described (22). The following globin-specific probes were used: mouse ϵ , pSP65M ϵ (23); mouse β^{maj} , pSP64M β 134 (23); mouse β h1, pSP65 β h1 (7); human γ , pT7 γ^{m} (19).

Globin-Chain Analysis. Single-cell suspensions of liver or peripheral blood were divided into three aliquots. One aliquot was used to prepare cytological smears for labeling with anti- γ -globin monoclonal antibodies (24). Another was used directly for isoelectric focusing of globin chains, and the last aliquot was incubated in the presence of 75 μ Ci (1 Ci = 37 GBq) of [3 H]leucine prior to isoelectric focusing. Following focusing, nonradioactive gels were stained with Coomassie blue. Radioactive gels were fixed, dried, and fluorographed (22).

RESULTS

Production of μ LAR- γ Transgenic Mice. Forrester *et al.* (14) have recently made a 2.5-kb fragment containing all four of the 5' developmentally stable hypersensitive sites. This fragment, termed μ LAR, confers high-level expression on a transfected human β -globin gene in mouse erythroleukemia cells. We linked μ LAR to a 3.3-kb γ -globin-gene-containing fragment. This construct, μ LAR- γ , is illustrated in Fig. 1.

The eukaryotic insert was purified from vector sequences and injected at a concentration of 1 μ g/ml into 60 fertilized mouse eggs. Twenty eggs were transferred into the uteri of each of three pseudopregnant foster mothers. Two of these mice became pregnant, and embryos were removed from them after 14 days of development. Fourteen embryos were obtained. Duplicate slot blots of embryonic brain DNAs were analyzed by using probes specific for the -18 and -15 hypersensitive sites and the γ exon 3 regions. Six embryos containing both LAR and γ -globin sequences were identified. DNAs prepared from the carcasses of these transgenic embryos and from the carcass of a nontransgenic littermate were further analyzed by Southern blotting. DNAs were restricted with the following enzymes: *Pst* I, *Bcl* I and *Bam*HI, or *Bcl* I and *Sph* I. Blots were hybridized with a probe containing the 5' flanking and genic regions up to exon 2 from the γ -globin gene (see Fig. 1). Fragments consistent

FIG. 1. Map of μ LAR- γ . The developmentally stable hypersensitive sites are shown in open boxes and are designated by their native positions relative to the human ϵ -gene. The γ -globin gene fragment is indicated by a solid line and its exons are shown as solid boxes. The open box under the diagram indicates the location of the probe used for Southern blot analysis. The restriction sites used for Southern blot analysis and release of the fragment from the vector are shown. N, *Not* I; B, *Bcl* I; P, *Pst* I; M, *Bam*HI; H, *Sph* I; S, *Sal* I.

with the presence of 1 to 11 unrearranged copies of the transgenic construct were obtained in all 6 mice.

Expression at the RNA Level. RNA was prepared from the blood, brains, and livers of 14-day transgenic embryos and a nontransgenic littermate. At this stage of development, the fetal liver is the primary site of definitive erythropoiesis, and fetal liver erythroid cells produce only adult globin. Fourteen-day embryo blood, on the other hand, is composed of non-nucleated definitive erythrocytes released from the fetal liver, together with nucleated primitive erythroid cells persisting from yolk sac-derived erythropoiesis (see refs. 5 and 7).

Blood and fetal liver samples were analyzed for the presence of human γ -globin mRNA by RNase protection mapping with a probe that protects exons 1 and 2 of the globin message (Fig. 2). Correctly initiated γ -globin mRNA was detected in the blood and livers of all the transgenic embryos but not in the nontransgenic animal. The level of expression in the two tissues was consistent for any given mouse but varied considerably between individual mice. To see if γ -globin gene expression was erythroid specific, we performed a similar protection analysis on brain RNA (Fig. 3). To control for possible blood contamination of the brain, we simultaneously probed for mouse β^{maj} mRNA. Blood from one of the transgenic animals was used as a positive control. Both mouse and human messages were detected in the blood control but not in any of the transgenic brain RNAs, indicating that the expression of the transgene was erythroid specific.

There are still embryonic erythrocytes circulating at this stage of murine development, so there is a possibility that γ expression in the transgenic livers was derived from persistent circulating embryonic erythroid cells contaminating the liver. We ruled this out by analyzing the fetal liver samples for the presence of the murine β -like globin embryonic messages β h1 and ϵ^y (Fig. 4). High levels of both these messages can be found in 11.5-day mouse embryo blood, so blood RNA from an 11.5-day nontransgenic animal was used as a positive control. β h1 RNA was not detected in either the blood or liver. Low levels of ϵ^y mRNA [the last embryonic gene to be silenced during mouse development (7, 22)] could

FIG. 2. RNase protection analysis of γ -globin mRNA expression. Fifty nanograms of RNA prepared from fetal liver (A) or blood (B) was analyzed for the presence of γ -globin mRNA. In both A and B, lanes 1-6 contain samples from transgenic animals designated A5, A6, B2, B4, B5, and B6, respectively; lane 7 contains RNA from a nontransgenic mouse designated A1. The probe protects fragments of 138 and 204 nucleotides corresponding to exons 1 (ex.1) and 2 (ex.2) of the γ -globin message, respectively. Lane M, *Hpa* II-cleaved pGEM (Promega) DNA molecular size markers.

FIG. 3. Tissue specificity of γ -globin transcription. One hundred nanogram portions of RNA were simultaneously analyzed with probes for exon 2 of the human γ -globin transcript (204-nucleotide protected band) and exon 1 of the mouse β^{maj} message (134-nucleotide protected band). Lane 1, blood RNA from a nontransgenic control mouse, A1. Lane 2, blood RNA from a transgenic mouse, B5. Lanes 3–8, brain RNAs from transgenic embryos A5, A6, B2, B4, B5, and B6, respectively. Lane 9, tRNA control.

be found in the blood but not the liver samples. Since no mouse embryonic RNA could be detected in any of the transgenic fetal liver RNAs, we concluded that the expression of the transgene was truly definitive in nature.

The level of γ -globin transcription in the fetal liver was measured by simultaneously analyzing samples with a probe specific for the exon 2 region of the human γ -globin transcript and another for exon 1 of the endogenous mouse β^{maj} -globin

FIG. 4. Analysis of murine embryonic globin expression. RNAs (100 ng) from the liver (lanes 1–3) and blood (lanes 6–8) of transgenic embryos were simultaneously analyzed for the presence of β^{h1} (182-nucleotide protected product), ϵ^{y} (145-nucleotide protected product), and human γ (204-nucleotide protected product) messages. Lanes 4 and 5, 100 ng and 5 ng of 11.5-day nontransgenic mouse embryo blood, respectively. Lane M, *Hpa* II-cleaved pGEM DNA molecular size markers.

FIG. 5. (A) Copy number analysis. *Bcl* I/*Bam*HI-digested carcass DNAs were blotted and analyzed with a γ -globin-specific probe. Lanes 1–6, DNAs from transgenic mice A5 (8.7), A6 (10.9), B2 (6.9), B4 (1), B5 (4.9), and B6 (2.4), respectively. Numbers in parentheses indicate the approximate copy number of the transgene. (B) Level of γ -globin expression in the fetal liver. One hundred nanogram portions of liver RNA were simultaneously analyzed for human γ - and mouse β^{maj} -globin transcripts as described in the legend to Fig. 3. Lanes 1–6, RNAs from transgenic mice A5, A6, B2, B4, B5, and B6, respectively.

message (Fig. 5B). The steady-state level of γ -globin ($\gamma/(\gamma + \beta^{\text{maj}})$) mRNA ranged from 5% to 18%. However, when these values are corrected for gene copy number (Fig. 5A), they range from 2.8% to 10% with a mean value of $5.5 \pm 2.2\%$.

Expression at the Protein Level. The expression of the γ -globin chain in transgenic blood and fetal liver was documented at three levels—namely, synthesis (Fig. 6), accumulation (Fig. 7), and cellular distribution (Fig. 8). γ -Globin chain synthesis and accumulation were assessed by isoelectric focusing of [^3H]leucine-labeled and unlabeled globin chains, respectively. Examples from the liver and blood of two transgenic embryos and a nontransgenic control are shown in Figs. 6 and 7. The liver samples contain predominantly adult mouse globin chains, whereas the blood samples contain both adult and embryonic globins (Fig. 7). The latter derive from the remaining primitive erythroid cells in the

FIG. 6. Globin chain synthesis. Single-cell suspensions of liver and blood were washed, incubated with [^3H]leucine, and lysed, and globin chains were analyzed by isoelectric focusing followed by autoradiography. Mouse chains X, Y, and Z derive from the ζ , ϵ^{y} , and β^{h1} genes, respectively. Lanes: 1, A5 liver; 2, A5 blood; 3, B2 liver; 4, B2 blood; 5, A1 (nontransgenic) liver; 6, A1 (nontransgenic) blood; cb, human cord blood.

FIG. 7. Isoelectric focusing of globin chains. Cells from the liver and blood of two transgenic and one nontransgenic animal were lysed. Globin chains were analyzed by isoelectric focusing and visualized by Coomassie blue staining. Globin chain designation is as in Fig. 6. Lanes: 1, A5 liver; 2, A5 blood; 3, A1 (nontransgenic) liver; 4, A1 (nontransgenic) blood; 5, B2 liver; 6, B2 blood; cb, human cord blood (to indicate the mobility of the γ -globin chain).

bloodstream. The fact that these late cells are not very active in globin synthesis explains why these embryonic globins are not as apparent in the isoelectric focusing of blood globin chains labeled *in vivo* by the incorporation of tritiated leucine

FIG. 8. Immunofluorescent labeling with fluorescein isothiocyanate-conjugated anti- γ -globin of cytological spreads from fetal liver and blood of transgenic mice. (A) A virtually homogenous population of fetal liver erythroblasts. Note that only a proportion of cells appear intensely labeled. (B) A cluster of circulating embryonic erythroblasts. Approximately one-third of the embryonic erythroblasts are positively labeled. (C) Mature erythrocytes from blood. The large intensely labeled cells arise from definitive fetal erythropoiesis. The small unlabeled cells are contaminating maternal erythrocytes.

Table 1. Hemoglobin chains accumulated in the blood and liver of μ LAR- γ transgenic fetuses

Fetus	Tissue	% non- α -hemoglobin chains		
		Mouse		Human γ
		Embryonic	Adult	
A5	Blood	33.7	58.6	7.7
	Liver	9.0	72.4	18.6
A6	Blood	33.1	64.8	2.1
	Liver	0	90.3	9.5
B2	Blood	31.8	58.8	9.9
	Liver	0	91.7	8.3
B4	Blood	41.7	55.5	2.7
	Liver	0	94.3	5.7
B5	Blood	44.4	46.0	9.6
	Liver	0	91.4	8.6

(Fig. 6). γ -Globin is clearly present as well as being actively synthesized in both the blood and fetal liver.

The quantitation of hemoglobins accumulated in the blood or liver of the transgenic fetuses is presented in Table 1. The blood samples contain substantial amounts of embryonic hemoglobins (from 32% to 44% of total hemoglobin) due to presence of yolk sac erythrocytes. In spite of this, the amount of human γ chain in these blood samples is either similar to (fetuses B2 and B5) or lower than (fetuses A5, A6, and B4) the amount of γ -globin in the fetal liver samples of the same fetuses. These results suggest that there is no preferential expression of the human μ LAR- γ during the yolk sac stage of murine erythropoiesis.

The cellular distribution of γ -globin production was investigated by staining cytocentrifuge-prepared cellular spreads of fetal liver and blood cells with fluorescent-conjugated monoclonal antibodies specific for the human fetal globin chain (24). The results shown in Fig. 8 are typical of those obtained. Fig. 8A shows γ -globin expression in definitive fetal liver erythroid cells. γ -Globin was also detected in both the primitive nucleated (Fig. 8B) and definitive nonnucleated erythroid cells (Fig. 8C) present in the embryonic blood. As in the liver, the expression appeared heterocellular. It seemed unlikely that this was the result of chimerism since it was observed in all six transgenic embryos. To address this possibility, we performed DNA slot blot analysis of fetal liver and brain DNA from two transgenic embryos. We found that the copy number of the transgene was the same in both tissues (data not shown), suggesting that these were not chimeric mice.

Expression in the Adult Animal. In another experiment, we injected 60 fertilized eggs with μ LAR- γ . These eggs were transferred into the uteri of three pseudopregnant mothers (20 eggs each). Nine liveborn mice were obtained. They were tested at 14 days of age, and one of these was found to be transgenic and to express the human γ -globin gene. γ -Globin accounted for 33% of total non- α -chains, and its distribution appeared to be heterocellular (Fig. 9).

DISCUSSION

LAR and Globin Gene Switching. Our experiments show that the LAR- γ construct is expressed in the embryonic globin-expressing primitive erythroid cells of the mouse and also in adult globin-expressing definitive erythroid cells. Since the fetal globin genes are not normally expressed in adult cells, these data suggest that other sequences in the globin locus are important for the developmental regulation of γ -globin genes during development. One explanation for our results is a position effect. *In vivo* the LAR is separated from the γ -globin genes by about 20 kb. In μ LAR- γ the intervening 20 kb has been removed, and the LAR is juxta-

FIG. 9. Immunofluorescent labeling (with anti- γ -chain monoclonal antibody) of the blood of an adult μ LAR- γ transgenic mouse demonstrating expression of the human γ -gene in the adult animal.

posed to the γ -globin gene. At this short range, the LAR may act as an enhancer, whereas *in vivo* it might act quite differently. Alternatively, there may be sequences in the locus that specifically counter the LAR effect and silence the γ -globin genes in adult life. Clues as to the location of these sequences may be provided by a group of hereditary mutations in humans characterized by the persistence of fetal globin expression in adult erythroid cells (HPFH). One class of HPFH mutations involves deletions of the 3' region of the cluster, which remove the δ and β genes but leave the γ and α genes intact (see ref. 1). By systematically adding back candidate sequences to the μ LAR- γ construct and screening expression in primitive and definitive transgenic erythroid cells, it should be possible to determine the region that is responsible.

Therapeutic Potential of Definitive γ -Globin Expression. Attempts at gene therapy using hematopoietic cells have focused on introducing adult globin genes. However, in the case of sickle cell anemia, introduction and expression of the human fetal globin gene would be more desirable, because fetal globin is a far more potent anti-sickling agent than normal adult β -globin (25). The fact that fetal globin genes are not expressed in adult cells presents a major obstacle. However our results show that the LAR can be used to respecify the developmental program of a fetal globin gene and thereby allow its expression in adult cells. Although the absolute amount of hemoglobin F per cell required to successfully ameliorate sickle cell anemia is quite low (approximately 20%), it is necessary that hemoglobin F be formed in most of the erythroid cells of the bloodstream [i.e., that expression be pancellular (25)]. Our analyses of the cellular distribution of μ LAR- γ globin in transgenic mice by using anti- γ -globin monoclonal antibodies demonstrate that expression is heterocellular. Heterocellular expression does not appear to be the result of chimerism. It may, on the other hand, be a consequence of the relatively low level of γ expression we observed. Our comparative analyses of γ -globin accumulation in the blood and fetal liver suggest that low-level γ -globin expression is not a peculiarity of definitive erythropoiesis but is also a feature of the yolk sac stage. Possibly the interaction of the LAR with the γ -globin gene is not as efficient as with the β -globin gene. Alternatively, sequences deleted from the genomic LAR region (19.5 kb) to produce μ LAR (2.5 kb) may be important for pancellular

expression. Finally, γ -gene sequences in addition to the 3.3-kb *HindIII* fragment may be needed for high level γ -globin expression. Further studies on the level and cellular distribution of γ -globin expression in more γ -globin-LAR recombinants will be needed to address these possibilities.

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