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Human α-Globin Genes Demonstrate Autonomous Developmental Regulation in Transgenic Mice

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Recent studies have demonstrated that transcriptional activation of the human adult β-globin transgene in mice by coinsertion of the β-globin cluster locus control region (β-LCR) results in loss of its adult restricted pattern of expression. Normal developmental control is reestablished by coinsertion of the fetal γ-globin transgene in *cis* to the adult β-globin gene. To test the generality of this interdependence of two globin genes for their proper developmental control, we generated transgenic mice in which the human adult α-globin genes are transcriptionally activated by the β-LCR either alone or in *cis* to their corresponding embryonic ζ-globin gene. In both cases, the human globin transgenes were expressed at the appropriate developmental period. In contrast to the β-globin gene, developmental control of the human adult α-globin transgenes appears to be autonomous and maintained even when activated by an adjacent locus control region.

The hemoglobin molecule is a tetrameric metalloprotein containing two α-like globin chains and two β-like globin chains (8, 43). The human α-globin gene cluster (Fig. 1, top) includes four functional genes (5′ to 3′, ζ, α2, α1, and Θ) and three pseudogenes (ψζ, ψα2, and ψα1) encoded on chromosome 16 (13, 34). The human β-globin gene cluster includes five functional genes (5′ to 3′, ε, ^{G}γ, ^{A}γ, δ, and β) and one pseudogene (ψβ), encoded on chromosome 11 (8, 12, 20). The genes within each cluster are temporally regulated and are organized in the order of their developmental expression (8, 43), with embryonic genes 5′-most and adult genes 3′-most. In the human β-globin cluster, the ε-globin gene is expressed in the first 7 weeks of gestation, followed by the expression of the two adjacent γ-globin genes during the fetal period (7 weeks to term) and finally by the adult β- and δ-globin genes at birth (24, 35). In contrast, only one switch takes place in the α-globin cluster, embryonic (ζ) to fetal/adult (α2 and α1) at 6 to 7 weeks of gestation (35). The Θ-globin gene is expressed at low levels of mRNA, in parallel with the α1- and α2-globin mRNAs (1, 28; unpublished data).

The organization and developmental switching patterns of the human globin genes provide a model system for the study of gene expression. In addition, understanding globin gene regulation is clinically relevant to therapy of genetic diseases such as thalassemia and sickle cell anemia (8, 43). Recent experiments in which specific segments of the β-globin gene cluster have been introduced into the mouse germ line have substantially advanced the understanding of the developmental switching of globin gene expression. Results from these experiments suggest that when the γ- and β-globin genes are carried in transgenic mice as isolated gene fragments and expressed at low levels, the sequences in their immediate vicinity are sufficient to establish normal developmental control (embryonic and fetal/adult) (3, 9). Achievement of full levels of γ- and β-globin transgene expression is dependent on the cointegration of a DNA segment encompassing a series of DNase-hypersensitive regions located 5′ of the native β-globin gene cluster (19, 21, 38). This locus control region (LCR) has been previously referred to as the dominant control region (19) or locus activating region (16, 38). A region with similar properties has recently been localized 5′ to the human α-globin cluster (23). When the γ- and β-globin transgenes are independently juxtaposed to the β-globin cluster LCR (β-LCR), their developmental control is lost and they are expressed throughout development. Their appropriate expression is reestablished when they are positioned in their native 5′-to-3′ orientations in *cis*. These data suggest that the adult and embryonic β-globin genes must be positioned in a way that allows them to compete for some LCR-related activity to maintain their normal developmental control (4, 16).

An intrinsic problem in studying the developmental control of the human β-globin genes in the mouse is that mice and humans have distinctly different developmental patterns of β-globin gene switching. The mouse β-globin system, unlike its human counterpart, lacks a fetal-stage-specific gene (9). Instead, the mouse β-globin cluster contains three embryonic β-like globin genes (εY, βh0, and βh1) whose expression is replaced by two fetal/adult genes (β^{maj} and β^{min}) at 11 days of gestation (embryonic-to-fetal/adult switch). The lack of fetus-specific *trans*-acting factors in the mouse raises concerns about interpreting the results of the human ^{A}γ- to β-globin transgene developmental switch. In contrast, the organization and developmental regulation of the mouse and human α-globin clusters are identical (17, 22, 27, 29); a single embryonic ζ-globin gene switches to two coexpressed fetal/adult α-globin genes (α2 and α1). This parallel pattern of developmental control and structural similarity between the α-globin genes of mice and humans suggests that study of the human α-globin cluster in the transgenic mouse may serve as a more appropriate model for the study of globin gene regulation and developmental switching. In this study, we tested the hypothesis that the ζ-globin gene is required to be linked in *cis* to the α-globin gene for normal developmental switching to proceed.

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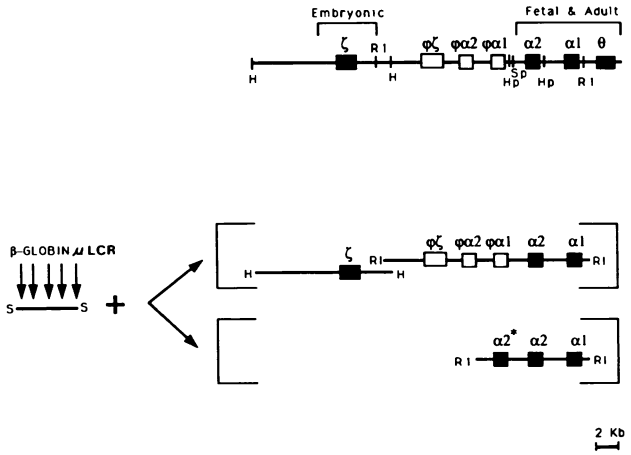

FIG. 1. Fragments of human α-globin cluster injected into fertilized mouse eggs. The human α-globin gene cluster is shown on the top line; genes and pseudogenes are represented by solid and open boxes, respectively. The developmental stages at which these genes are normally expressed are indicated. Selected restriction sites are indicated: RI, *Eco*RI; Sp, *Sph*I; H, *Hin*dIII; Hp, *Hpa*I; S, *Sst*II. The two sets of DNA fragments coinjected into fertilized mouse eggs are shown on the second and third lines. The β-globin LCR (β-LCR) contains all five DNase I-hypersensitive sites on a 6.5-kb *Sst*II fragment (44). This fragment was coinjected with the 23-kb *Eco*RI fragment containing the two α-globin genes (α2 and α1) and three pseudogenes and the 13-kb *Hin*dIII fragment containing the functional ζ-globin gene (ζ2) (top bracket) or with the 12-kb fragment containing only the two normal adult α-globin genes and a marked α2-globin gene (*; expression of this gene was not studied) (bottom bracket).

MATERIALS AND METHODS

Isolation of DNA fragments containing segments of the human α-globin gene cluster. The 23-kb *Eco*RI fragment of the α-globin cluster extending from 5′ of the ψζ-globin gene to 3′ of the α1-globin gene was subcloned from cosmid pCL9 (26) into the *Eco*RI site of pSP64 (Promega). The 13-kb *Hin*dIII fragment containing the ζ-globin gene was similarly subcloned from pCL9 into pSP64. The 12-kb *Eco*RI fragment containing the two adult α-globin genes (last line of Fig. 1) also contains a marked α2-globin gene (α*) at the position normally occupied by the ψα-globin gene. This marked gene was inserted for a series of experiments unrelated to the present study.

The 12-kb fragment containing the adult α-globin genes was assembled in several steps. The pCL9 cosmid was first digested with *Sph*I, which cuts 2.5 kb 5′ to the α2-globin gene. The *Sph*I ends were filled in with DNA polymerase I (Klenow fragment) and ligated to linkers containing an exposed cohesive *Eco*RI site, followed by an internal *Not*I site and a blunted end. After linker ligation, this fragment was digested with *Eco*RI, which cuts 3′ to the α1-globin gene. The released 8-kb fragment was ligated to the *Eco*RI-linearized pSP64 vector. This recombinant plasmid was then linearized at the *Not*I site (upstream of the α2-globin gene), and a 4.2-kb *Hpa*I genomic fragment containing a marked α2-globin gene (see below) and adapted with *Not*I ends was inserted at this position. The 4.2-kb *Hpa*I α2-globin gene fragment (α*) was marked by the insertion of a *Pvu*II-*Hin*dIII oligonucleotide adapter at the *Hin*dIII site internal to exon 2 of the gene. All three α-globin genes were in the same transcriptional orientation, as determined by restriction mapping. The β-LCR construct used in these studies, p1417 (44), contains all five DNase I-hypersensitive sites within a 6.5-kb *Sst*II fragment.

Generation of transgenic mice. Each of the DNA fragments to be injected (Fig. 1, bottom) was first released from its plasmid vector. The β-LCR was released by digestion with *Sst*II, the ζ-globin gene was released by digestion with *Hin*dIII, the 23-kb fragment containing the α-globin genes was released by digestion with *Eco*RI, and the 12-kb insert containing the two normal α-globin genes and the marked α2* gene was released by *Eco*RI digestion.

The digests were run on an agarose gel, and the regions of the gel containing each of the fragments were visualized by ethidium bromide fluorescence and excised. The DNA was recovered from the gel slices by adsorption to glass beads (41). The amounts of each fragment which were coinjected were adjusted so as to be equimolar. On average, we injected 2 to 3 pl of 2-, 2-, 4-, and 3-ng/μg solutions of β-LCR, ζ, α2α1, and α2α2α1, respectively, per egg. DNA was microinjected into the male pronucleus of either fertilized F_2 hybrid eggs from C57BL6 × CBA or fertilized F_1 hybrid eggs from SWR × SJL mice as described previously (7). The injected eggs were incubated in Whitten's medium at 37°C overnight, followed by transfer into the oviduct of a CD-1 pseudopregnant foster mother. The day when the vaginal copulation plug was observed was considered day 0.5. Hemizygous embryos were generated by mating each of the founders to normal SWR or (C57BL6 × CBA)F_1 females.

DNA analysis. DNA preparation and Southern blotting were performed as described previously (41). Restriction enzyme and probe specifications are given in Table 1, footnote *a*. Final blot washes were carried out in 0.1× SSC–0.5% sodium dodecyl sulfate for 30 min at 65°C (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate).

RNA analysis by RT/PCR. Human reticulocyte RNA was isolated from acid-precipitated polysomes of an individual with sickle cell anemia and a normal α-globin genotype (described in reference 1). Mouse reticulocyte RNA was prepared from phenylhydrazine-treated adult mice (5) and from total embryos by the guanidine hydrochloride method (1). The concentrations were determined by A_{260} measurements. Primers used for reverse transcription and cDNA amplifications were synthesized by the DNA synthesis facility of the Cancer Center at the University of Pennsylvania. In each reverse transcriptase-polymerase chain reaction (RT/PCR) experiment, one of the primers in each set was end labeled with [γ-^{32}P]ATP (Amersham; 5,000 Ci/mmol) in the presence of T4 polynucleotide kinase (New England BioLabs) by a standard protocol (41). All primers were labeled to the same specific activity. Reverse transcription was carried out under conditions optimized for full-length globin cDNA synthesis (1) with avian myeloblastosis virus RT (Life Sciences, St. Petersburg, Fla.). The reaction mix was phenol extracted, ethanol precipitated, dried, and taken up in 25 μl of water prior to PCR. PCR was carried out as described previously with the following cycling conditions: initial denaturation at 94°C for 10 min, followed by 30 successive cycles of renaturation at 58°C for 30 s, polymerization at 74°C for 4 min, and denaturation at 94°C for 1 min. The primers used for RT/PCR of the human α- and ζ-globin mRNAs were those described previously (1). The 5′ and 3′ primers used in the RT/PCR reaction of the mouse α-globin mRNA correspond to codons 28 to 35 (5′CCTGGAAAGG ATGTTTGCTAG3′) and the 3′ mRNA terminus (5′TTTTTT TTTGCAGGCTTC3′), respectively. The 5′ and 3′ primers used in the RT/PCR reaction of the mouse ζ-globin mRNA

correspond to codons 28 to 37 (5'CTAGAGAGGCTCTTC TGCAGCTACCCC3') and to nucleotides 12 to 39 upstream of the poly(A) tail (5'GATCATAGCTGGTCATGGGGGT CG3'), respectively. The 3' primer which was used for RT/PCR of the mouse β-globin mRNA (5'AGTGGCCACT CCAGCCACCAC3') corresponds to codons 121 to 128 of the mouse β^{min}-globin gene (25). The 5' primer, complementary to both mouse β^{min}- and β^{maj}-globin mRNAs (GCCCTG GGCAGGCTGCTGGTTG), bridges the exon 1- and exon 2-encoded segments (25). The ability to accurately quantitate changes in the relative levels of human α- and mouse β-globin mRNAs or of human α- and human ζ-globin mRNAs by this assay was demonstrated by RT/PCR analysis of serial mixtures of RNA samples containing either human α- and ζ-globin mRNA (1a) or human α- and mouse β-globin mRNA (see Fig. 3).

RNA analysis by primer extension. Primer extension analysis of human α- and mouse β-globin mRNAs was carried out with 5'-end ^{32}P-labeled primers (see above). The two primers were labeled to equal specific activities (3×10^6 cpm/μg). The primer extension reaction mixes contained unlabeled mouse α-globin oligonucleotide corresponding to the position of the human α-globin primer to eliminate potential cross-hybridization to the endogenous mouse α-globin mRNA. Amplification and primer extension products were analyzed on an 8% polyacrylamide–8 M urea gel and directly autoradiographed. Band intensities were quantified by soft laser densitometry (Molecular Dynamics 300A computing densitometer).

Hemoglobin analysis. Peripheral blood was obtained from the tails of adult mice or by crushing total embryos. The crushed 11-day embryos contained few erythroid cells, and several attempts were carried out to obtain sufficient globin for detection by direct staining in analytic gels. The blood was washed three times in 0.9 M NaCl and subsequently osmotically lysed in water. Then, 3 μl of each clarified lysate was analyzed by isoelectric focusing electrophoresis on an LKB Ultrophor apparatus (30) or by Triton-acid-urea gel electrophoresis (2). Proteins on all isoelectric focusing and Triton-acid-urea gels were visualized by staining with 1% Coomassie brilliant blue (30).

RESULTS

Generation of mouse lines expressing high levels of human α-globin mRNA. Isolated human α-globin genes are not expressed in transgenic mice (21, 40). However, high levels of human α-globin gene expression can be achieved if the α-globin gene is juxtaposed with the β-LCR (21, 40). To establish mouse lines with functioning human α-globin genes, we therefore coinjected the β-LCR into the male pronucleus of fertilized mouse eggs along with human genomic fragments containing one or more segments of the α-globin gene cluster (Fig. 1). Such coinjected fragments usually integrate at a single locus in the oocyte genome (7). The β-LCR fragment used contains all five 5' DNase I-hypersensitive sites grouped on a 6.5-kb *Sst*II fragment (p1417) (generous gift of F. Grosveld [44]) (Fig. 1) and facilitates high-level expression of juxtaposed genes in erythroid tissue in an orientation- and position-independent manner (44).

In the first set of experiments, a 23-kb *Eco*RI fragment containing the α1- and α2-globin genes, a 13-kb *Hin*dIII fragment containing the ζ-globin gene, and the β-LCR were coinjected in equimolar concentrations (Fig. 1, second line). The first two fragments span the majority of the α-globin gene cluster, excluding only the Θ-globin gene. Analysis of human globin mRNA expression in mice carrying this combination of genes would establish whether adjacent human ζ- and α-globin transgenes are expressed in the mouse at the appropriate embryonic and adult stages. In a second set of experiments, the β-LCR was coinjected with an equimolar concentration of a 12-kb fragment containing only the adult-specific α-globin genes (Fig. 1, third line). Analysis of human mRNA expression in mice carrying these fragments would establish whether the adult α-globin gene is appropriately expressed in the absence of an adjacent embryonic ζ-globin gene.

TABLE 1. Transgene copy number[a]

Mouse line	No. of copies		
	α-Globin	ζ-Globin	β-LCR
1	3	5	4
2	2	—[b]	5
3	10	—	4
4	13	—	5
6	4	6	6

[a] Transgene copy number was determined by densitometric comparison of band intensities on Southern blots containing equal quantities of total genomic DNA from each of the lines and of normal human leukocyte DNA. For analysis of the α-globin, ζ-globin, and LCR fragments, the DNAs were digested with *Hin*dIII and blots were successively hybridized with an α-globin gene probe (760-bp *Sma*I-*Pvu*II fragment extending from IVSI to the 3'-flanking region), a ζ-globin gene probe (1,753-bp *Hin*fI fragment extending from IVSI to the 3'-flanking region), and a human β-LCR probe (1.4-kb *Hin*dIII fragment isolated from p1417 [44]), respectively.

[b] —, not present in injected DNA.

The number of transgenic embryos (13.5 days) and live-born mice was assessed from the two sets of injections. Twenty-three of 95 embryos and 6 of 85 live-born mice generated from eggs injected with these two sets of fragments were transgenic. The lower level of live-born transgenic mice may reflect a loss of fetuses with the highest levels of α-globin expression (19, 21). Two of the six founders (numbers 1 and 6) were derived from the first set of injections (α, ζ, and β-LCR), and the four remaining founders (2 through 5) were generated from the second set of injections (α and β-LCR). Each of the six founders was used to generate a continuous line of hemizygotes. As demonstrated by Southern analysis (data not shown), each line contained two or more copies of each of the coinjected fragments (Table 1). The pattern of hybridizing fragments was unique to each line and were co-inherited in an autosomal Mendelian manner.

Human α-globin mRNA expression in adult transgenic mice from each line. Reticulocyte RNA from the progeny of each founder mouse was initially analyzed for the presence of human α-globin mRNA by RT/PCR. In each amplification, one of the primers was ^{32}P-end labeled so that the cDNA products could be directly visualized by autoradiography of the analytic gel. The analyses of founders 1, 2, 3, and 4 are shown in Fig. 2. Each of the founders (including 5 [data not shown] and 6 [see Fig. 5]) expressed easily detectable levels of human α-globin mRNA. The specificity of the analysis was confirmed by *Hin*dIII restriction analysis, because *Hin*dIII cleaves human but not mouse α-globin cDNA (Fig. 2).

The level of human α-globin mRNA was compared with that of endogenous mouse α- and β-globin mRNAs in three of these lines (lines 1, 2, and 3). This comparison was carried out by two methods, RT/PCR and primer extension. In the RT/PCR analysis, the relative levels of human α-globin

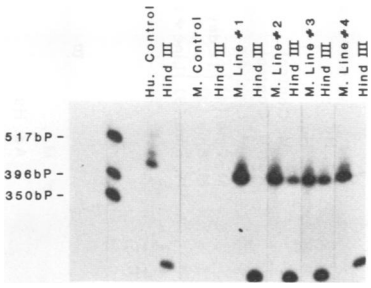

FIG. 2. Expression of human α-globin mRNA in four separate transgenic mouse lines detected by RT/PCR. Transgenic mouse lines generated from two sets of DNA coinjections (see Fig. 1) were screened for human α-globin mRNA expression by an RT/PCR assay. The 3′ primer used for both the RT and the PCR was ^{32}P end labeled, and the amplified cDNA products were directly detected by autoradiography of the analytic gel. Reticulocyte RNA samples assayed were isolated from the following sources: Hu. control, normal human; M. control, normal (nontransgenic) adult mouse; M. line 1 through M. line 4, adult mice from the four transgenic lines. An aliquot of each amplified cDNA was digested with *Hin*dIII and analyzed in the adjacent lane; *Hin*dIII will cleave human but not mouse α-globin cDNA (1, 17). The marker lane (M.) contains ^{32}P-end-labeled *Hin*fI-digested pG3. The sizes of the three marker bands are noted.

mRNA and endogenous mouse β-globin mRNA were analyzed in one tube, and in a parallel reaction the relative levels of endogenous mouse α- and β-globin mRNAs were compared. The human and mouse α-globin cDNA fragments which were amplified in the two separate reactions were the same size and had the same GC content and the primers were ^{32}P-end labeled to the same specific activities, all to ensure equal efficiencies of synthesis of the two α-globin cDNAs.

To demonstrate that the coamplified α- and β-globin cDNAs are detected in direct proportion to the starting concentrations of their respective mRNAs, we performed this RT/PCR analysis on mixtures containing known proportions of normal mouse (containing mouse β-globin mRNA) and human (containing human α-globin mRNA) reticulocyte mRNA preparations (Fig. 3A). The results demonstrate a linear correlation between the relative input of human α- and mouse β-globin mRNA and the relative yields of the respective cDNA products (Fig. 3B).

This assay was applied to the reticulocyte mRNA isolated from adults from lines 1, 2, and 3. The ratios of human α- to mouse β-globin mRNA in these three lines were 1.0, 0.2, and 0.2, respectively (Fig. 4A). RT/PCR of mouse α-globin and β-globin mRNAs in the same samples demonstrated a consistent ratio of 0.4 in each of the lines as well as in reticulocyte RNA isolated from a nontransgenic control mouse (Fig. 4B). Assuming approximately equal efficiency in amplification of the human and mouse α-globin cDNAs (see above), these data suggest that the ratios of human-to-mouse α-globin mRNA in lines 1, 2, and 3 are 2.5, 0.5, and 0.5, respectively [(Hα/Mβ) ÷ (Mα/Mβ)]. Lines 4 to 6 were not studied in similar detail.

To confirm the quantitation of α-globin transgene expression, we directly compared the absolute level of the human α-globin mRNA with that of the mouse β-globin by primer extension analysis (Fig. 4C). With human α- and mouse β-globin-specific primers labeled to the same specific activity, the ratio of human α-globin to mouse β-globin mRNA in lines 1 and 2 was 5.0 and 1.0, respectively. Since the

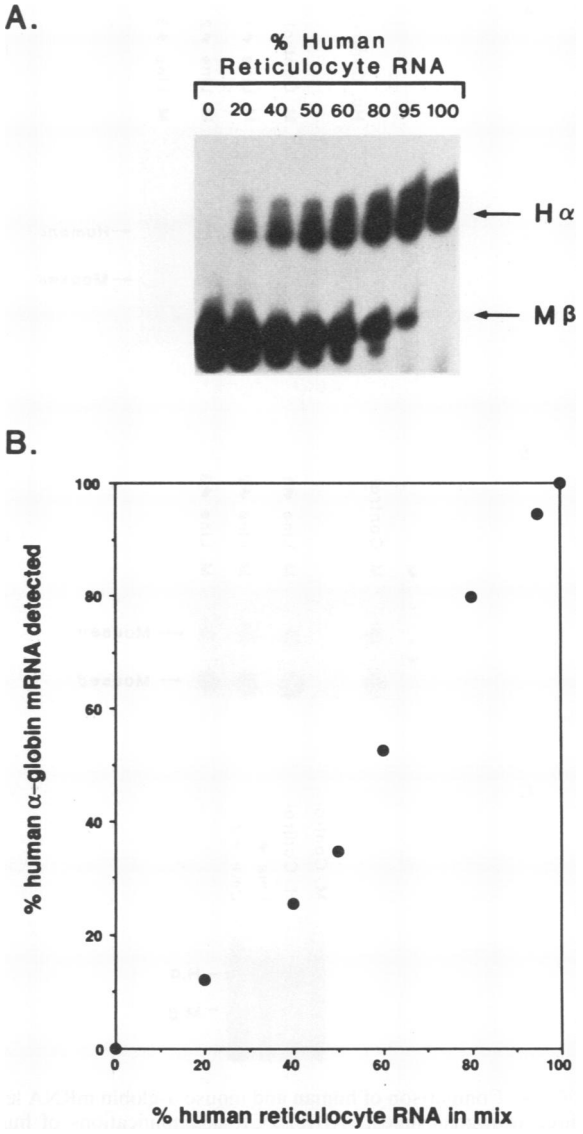

FIG. 3. Linearity of the RT/PCR coamplification analysis. (A) Human and mouse reticulocyte RNA samples were mixed at various ratios and analyzed by RT/PCR with two primer sets which will detect human α-globin (Hα) and mouse β-globin (Mβ) mRNAs (described in the text). The 5′ primer used for RT/PCR of the mouse β-globin mRNA and the 3′ primer used for RT/PCR of the human α-globin mRNA were both 5′-end ^{32}P-labeled primers. The percentage of human reticulocyte RNA present in each mix is indicated at the top of each lane, and the predicted positions of amplified human α- and mouse β-globin cDNAs are shown to the right of the autoradiograph. (B) The percentage of the human α-globin cDNA amplification products detected in each reaction mix is plotted on the ordinate against the percentage of human reticulocyte RNA present in the mix on the abscissa. The relative intensities of the bands in panel A were quantified by direct counting (Molecular Dynamics PhosphorImager).

concentration of α-globin mRNA exceeds that of β-globin mRNA by approximately twofold in normal reticulocytes (36), these data would equate to a human-to-mouse α-globin ratio of 2.5 and 0.5, respectively in these samples. Therefore, the results of the primer extension analysis and those of the RT/PCR coamplification assay demonstrate comparably

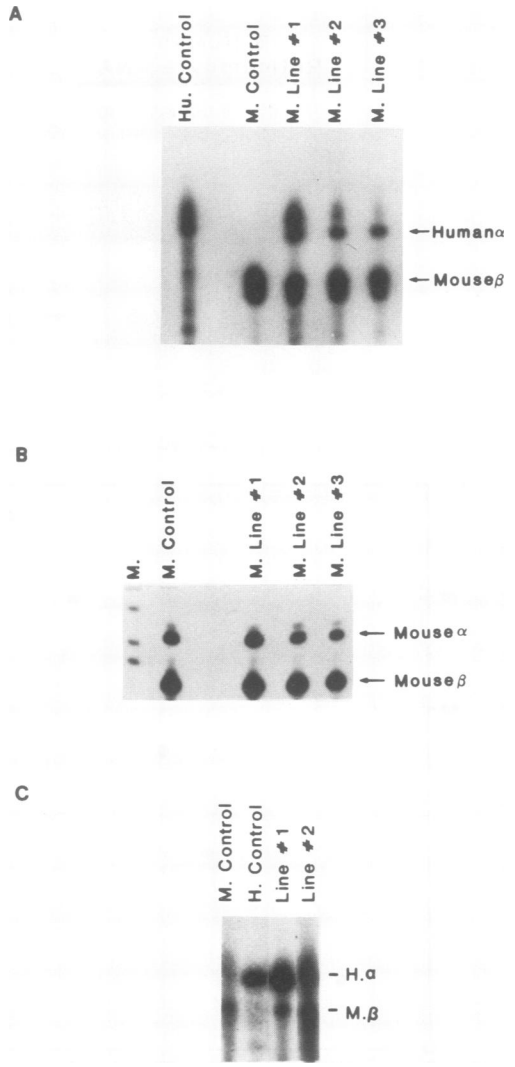

FIG. 4. Comparison of human and mouse α-globin mRNA levels in three transgenic lines. (A) RT/PCR coamplifications of human α-globin and mouse β-globin mRNAs. The two primer sets used in these reactions (detailed in the text and in Fig. 3) detect human α-globin mRNA (α2 and α1) and mouse β-globin mRNA (minor and major). The expected positions of the amplified human α- and mouse β-globin cDNAs are indicated. Lane designations are as detailed in the legend to Fig. 2. (B) RT/PCR coamplifications of mouse α- and β-globin mRNAs. The two primer sets are described in the text, and the expected positions of the amplified mouse α- and β-globin cDNA fragments are indicated. (C) Primer extension analysis of human α-(H.α) and mouse β-globin (M.β) mRNAs in reticulocyte RNAs. The priming oligonucleotides were ^{32}P end labeled. Lane designations are as above, and the positions of the full-length primer extension products of the human α- and mouse β-globin mRNAs are noted to the right.

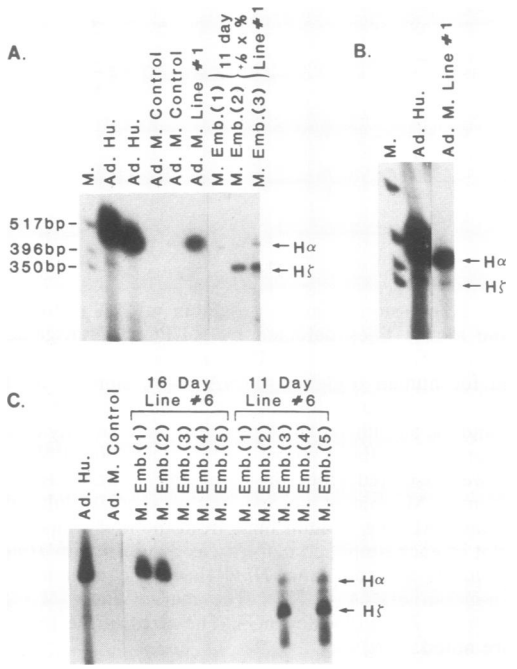

FIG. 5. Developmental switching of human α- and ζ-globin gene expression in transgenic mouse lines 1 and 6. (A) RT/PCR coamplification of human α- and ζ-globin mRNAs in line 1. Reticulocyte RNA from an adult transgenic mouse from line 1 and total RNA from 11-day-old embryos generated by crossing a line 1 adult (male) with a nontransgenic female (+/0 × 0/0) were analyzed alongside normal human and mouse reticulocyte RNA controls. Lanes are labeled as described in the legend to Fig. 2. The predicted positions of the human α- (Hα) and ζ-globin (Hζ) PCR cDNA products are indicated to the right. Lane M contains ^{32}P-end-labeled *Hin*fI-digested pGEM3 size markers. (B) Extended exposure of the adult human reticulocyte RNA control (Ad. H.) and the adult line 1 mouse (Ad. M. line 1) lanes of the gel in panel A. (C) RT/PCR analysis of human α- and ζ-globin mRNA expression in line 6. Total RNA was isolated from 11- and 16-day embryos (Emb.) generated by crossing the line 6 founder (male) with a nontransgenic female. Controls were carried out as in panel A.

high levels of mRNA expression from the human α-globin transgenes.

The hematological data of adult hemizygous transgenic mice from all six lines were normal for mean corpuscular volume, mean corpuscular hemoglobin concentration, and total hemoglobin compared with age-matched normal mice (data not shown). Hemizygous F_1 progeny generated by mating the founders to normal females were killed at 4 weeks of age and showed no significant difference in the size or weight of their spleens compared with nontransgenic littermates. These results suggest that despite the active expression of human α-globin mRNA, these transgenic mice do not show evidence of significant imbalance of globin protein expression.

Appropriate developmental switch from human ζ- to α-globin gene expression in transgenic mice. To determine whether the human ζ- and α-globin transgenes are developmentally regulated in the mouse, we analyzed their expression in two separate lines (1 and 6) which contain both genes adjacent to the β-LCR. The endogenous switch from embryonic to fetal/adult globin gene expression in the normal mouse occurs between days 12 and 14 of gestation (27). mRNA from day 11 embryos should therefore contain the embryonic globin mRNAs, while samples from day 16 of gestation and onwards would contain fetal/adult-expressed globin mRNAs. The founders of lines 1 and 6 were mated to nontransgenic females, and embryos were obtained at 11 and 16 days of gestation. mRNA from these embryos and from the respective adult founders was assayed for relative levels of human ζ- and α-globin mRNAs by RT/PCR coamplification. Of the samples from the day 11 embryos isolated from

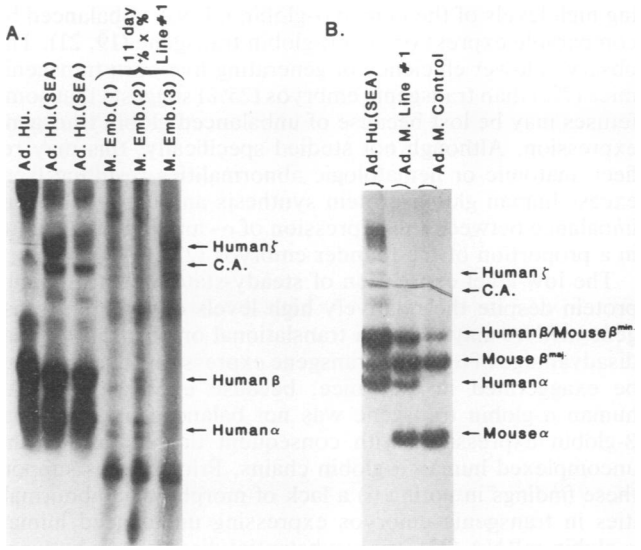

FIG. 6. Expression and developmental switching of the human α- and ζ-globin proteins in transgenic mice. (A) Detection of human ζ-globin chains in day 11 line 1 embryos. The embryos were generated by crossing the line 1 founder (male) with a nontransgenic female (+/0 × 0/0). Hemolysates from three embryos were analyzed in parallel with hemolysates from a normal human control and from a human heterozyous for the $(--)^{SEA}$ α-globin deletion (adults heterozygous for this deletion, which removes both fetal and adult α-globin genes from the cluster, express high levels of residual ζ-globin [11]). Globin content was analyzed by Triton-acid-urea-acrylamide gel electrophoresis. The positions of the human globin chains are indicated to the right of the gel. C.A., carbonic anhydrase. (B) Detection of human α-globin chains in an adult line #1 mouse. Hemolysate from the adult line #1 founder was analyzed in parallel with the controls noted in A. See legends to Fig. 2 and 5 for abbreviations.

both sets of matings (lines 1 and 6), 50% contained abundant levels of human ζ-globin mRNA (Fig. 5A and C; additional data not shown). Some of these samples contained trace amounts of human α-globin mRNA as well. In contrast, transgene mRNA analysis of samples at the fetal (day 16) and adult stages (lines 6 and 1, respectively) contain only α-globin mRNA (Fig. 5C and A, respectively). Residual expression of human ζ-globin mRNA can be detected in the adult line 1 mouse as well as in the normal human control after prolonged exposure of the analytic gel (Fig. 5B). This has been demonstrated previously for normal human reticulocytes (1). The human α-globin/ζ-globin mRNA ratio in the day 11 embryo of line 1 was 1:6, and in the adult founder it was 20:1. Comparison of these values demonstrates a 120-fold switch in the ratio of human α-globin and ζ-globin mRNAs during mouse development and a corresponding decrease in ζ-globin expression. Similar developmentally appropriate switching from human ζ- to α-globin transgene expression were obtained for line 6 (Fig. 5C).

Appropriate developmental switching from human embryonic ζ- to adult α-globin gene expression detected by protein analysis. To confirm the switch from human embryonic ζ- to adult α-globin gene expression by a second independent method, we directly assayed for switching at the level of hemoglobin composition. We were able to carry out this analysis in transgenic line 1, in which steady-state globin levels were high enough to be detected on stained protein gels (Fig. 6). Two of the 11-day embryos were negative for ζ-globin and were presumed to be negative for inheritance of the transgenes, while a third [M. Emb. (3)] contained substantial amounts of human ζ-globin (Fig. 6A). Analysis of the line 1 founder (Fig. 6B) demonstrated human α-globin and a complete absence of human ζ-globin. Comparable studies could not be carried out in line 6 owing to the lower levels of transgene expression.

Appropriate developmental control of isolated adult α-globin genes in transgenic mice. To determine whether appropriate developmental control of the human adult α-globin genes in lines 1 and 6 was dependent on the presence of the human embryonic ζ-globin gene in *cis*, we analyzed transgenic lines containing only the adult α-globin genes juxtaposed to the β-LCR. Four lines were generated, and two were selected at random for detailed study. A hemizygous transgenic male from line 2 was mated with nontransgenic females. Day 9 embryos, day 11 embryos, and adult progeny were analyzed for expression of the human α-globin mRNA by RT/PCR coamplification of human α- and mouse ζ-globin mRNAs. The endogenous mouse ζ-globin mRNA was coamplified as an internal control in each sample to confirm the quality of the embryonic RNA preparations. None of the 9-day embryos (total of 13 analyzed; 8 shown in Fig. 7 and an additional 5 not shown) contained human α-globin mRNA, while 50% of the 11-day embryos were positive for the human α-globin mRNA (Fig. 7A). The line 2 transgenic adults demonstrated high levels of human α-globin mRNA in the absence of mouse ζ-mRNA (for an example, see Fig. 7, last lane). Assay of day 9 embryos and adults from line 3 demonstrated an identical adult-specific pattern of α-globin transgene expression (data not shown). These results demonstrate that the α- and ζ-globin transgene switch occurs between 9 and 11 days of gestation. It is noted from the control coamplification of endogenous mouse α- and ζ-globin mRNAs in these samples (Fig. 7B) that there may be a minor discordance between the control of endogenous mouse α-globin mRNA, which is expressed at low levels as early as 9 days of gestation, and the human α-globin transgene mRNA, which appears to be more tightly controlled, appearing only after day 9.

DISCUSSION

The results of these studies demonstrate that normal developmental control of the human α-globin gene is not dependent on the presence in *cis* of the embryonic ζ-globin gene. In the first set of experiments, we generated two transgenic mouse lines (1 and 6) carrying DNA fragments encompassing most of the human α-globin gene cluster. Analysis of these two lines demonstrated developmentally appropriate expression of the human globin transgenes (Fig. 5). Switching from embryonic to adult α-globin synthesis occurs between days 11 and 16 of development. This parallels the switching of the corresponding endogenous mouse genes (Fig. 7B). The high level of human transgene expression in line 1 allowed us to confirm appropriate developmental switching by detecting replacement of the embryonic human ζ-globin with the adult human α-globin (Fig. 6).

To test whether the developmental regulation of the adult α-globin genes was dependent on the presence of the ζ-globin gene in *cis*, we generated transgenic lines which contained the human adult α-globin genes juxtaposed to the β-LCR in the absence of the embryonic ζ-globin gene. Data from two of these lines (2 and 3; lines 4 and 5 were not analyzed in detail) clearly demonstrated that human α-globin mRNA expression continues to be fetal/adult stage specific

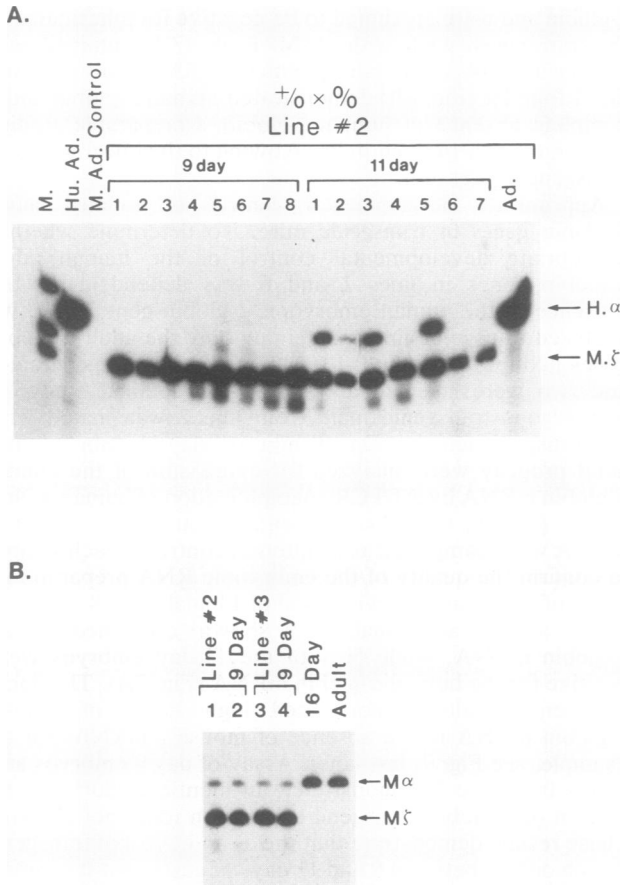

FIG. 7. Appropriate developmental control of isolated human adult α-globin genes in transgenic mice. (A) Analysis of human α- and mouse ζ-globin gene expression in the adult line 2 founder (Ad) and in 9- and 11-day embryos generated by the line 2 founder (+/0) with normal females (0/0). RT/PCR and coamplification of the human α- and mouse ζ-globin mRNAs were carried out as described in the text. The predicted positions of the amplified human α- (Hα) and mouse ζ- (Mζ) globin cDNA products are indicated to the right of the autoradiograph. Lanes are labeled as noted in Fig. 5. (B) RT/PCR coamplification of mouse α- and ζ-globin gene expression during development of line 2 mice. The mouse α- and ζ-globin mRNAs were RT treated and coamplified as detailed above. The RNAs were isolated from the reticulocytes of the line 2 founder (adult) and from day 9 and 16 embryos generated by crossing the founder with a nontransgenic female. Analysis of two 9-day embryos from lane 3 (which also fail to demonstrate α-globin expression) is also shown. The predicted positions of the two amplified cDNA products are indicated to the right of the autoradiograph.

in the absence of an associated ζ-globin gene (Fig. 7A). Human α-globin mRNA appeared to switch on between 9 and 11 days of development. We conclude from these data that appropriate developmental regulation of the human α-globin genes is independent of the presence of the ζ-globin gene. In a complementary set of experiments, Spangler and colleagues have demonstrated that the isolated human ζ-globin gene is appropriately developmentally regulated in transgenic mice when juxtaposed to the β-LCR (42). Therefore, it is apparent that both the ζ- and α-globin genes are autonomous in their developmental control.

When we began this study, it was unclear whether we would be able to generate live-born transgenic mice expressing high levels of the human α-globin mRNA unbalanced by comparable expression of a β-globin transgene (19, 21). The observed lower efficiency of generating live-born transgenic mice (7%) than transgenic embryos (25%) suggests that some fetuses may be lost because of unbalanced globin transgene expression. Although not studied specifically, this may reflect anatomic or hematologic abnormalities resulting from excess human globin protein synthesis and consequent net imbalance between the expression of α- and β-globin chains in a proportion of the founder embryos (23).

The low-level expression of steady-state human α-globin protein despite the relatively high levels of α-globin transgene mRNA may reflect a translational or posttranslational disadvantage of α-globin transgene expression. This gap may be exaggerated in our mice, because expression of the human α-globin transgene was not balanced by additional β-globin expression, with consequent degredation of the uncomplexed human α-globin chains. Prior studies support these findings in noting (i) a lack of morphologic abnormalities in transgenic embryos expressing unbalanced human α-globin mRNA (23), (ii) a substantial discrepancy between the levels of human α- and β-globin protein synthesis and the levels of the corresponding transgene mRNAs (18), and (iii) an inability to increase levels of human globin expression in mice by increasing the levels of the corresponding mRNAs (39). We demonstrate here that transgenic mice expressing relatively high levels of unbalanced human α-globin mRNA can be obtained and should provide a convenient model with which to study developmental regulation of the α-globin gene cluster.

The human embryonic and adult α-globin transgenes were activated by the β-LCR. A corresponding α-globin gene cluster LCR has recently been described (23) and is positioned comparably to the β-LCR (5′ to the cluster). The normal developmental control of the α-globin transgenes activated by the β-LCR which we observed in the present study suggests that the α- and β-LCRs are not only parallel in their positioning but may be parallel and possibly interchangeable in their function(s) as well (23).

The pattern of expression in transgenic mice of the human α-globin genes juxtaposed to the β-LCR contrasts with the results of similar studies carried out on the human $^{A}\gamma$- and β-globin genes (4, 15, 16). Both the $^{A}\gamma$- and β-globin genes are developmentally regulated in transgenic mice when they are introduced separately (4, 9, 15, 16, 33). They appear to lose their developmental regulation when each is linked to the β-LCR. The developmental control of these two genes is regained only when they are linked together (4, 16). These results suggest that the LCR overrides their temporal regulation in the absence of the native genomic organization (4, 16). Although these data are quite convincing, the interpretation is based on an assumption that in mice the human $^{A}\gamma$-globin gene behaves as an embryonic globin gene and the adult β-globin gene behaves as a fetal and adult gene (9). However, since the human γ- and β-globin genes are expressed at distinct stages of human erythroid development (fetal and adult, respectively) which do not occur in the mouse, these results may reflect a lack in the mouse of erythroid factors necessary for the human γ- to β-globin switch.

In contrast to the human β-globin gene cluster, the developmental control of the α-globin gene cluster in humans is parallel to that in the mouse. The autonomous developmental control of the α- and ζ-globin genes would predict that the organization of these genes within the cluster is not of critical importance to their timing of expression. The appro-

priate embryonic expression of the isolated human ε-globin transgene juxtaposed to the β-LCR (37) extends the finding of autonomous control to the only gene in the human β-cluster with a clear parallel in the mouse (embryo specific). These data suggest that the temporal regulation of the human α- and ζ-globin genes in the transgenic mouse is controlled by interaction between developmentally specific and evolutionarily conserved transcription factors that interact with signals in *cis* to the respective genes.

The structural similarity and the parallel developmental regulation of the mouse and human α-globin gene systems and the ability to maintain appropriate developmental regulation of the human α-globin cluster in mice upon juxtaposition to the β-LCR support the utility of this transgenic model of globin gene regulation and switching. Furthermore, the demonstrated ability to generate mice expressing substantial levels of human α-globin mRNA and detectable levels of α-globin protein may allow the development of model systems with which to explore the effects of excess and unbalanced globin synthesis on the function and development of the erythroid cells.

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