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Role of Gene Order in Developmental Control of Human γ- and β-Globin Gene Expression

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To determine the effect of gene order on globin gene developmental regulation, we produced transgenic mice containing two tandemly arranged γ- or β-globin or γβ- and βγ-globin genes linked to a 2.5-kb cassette containing sequences of the locus control region (LCR). Analysis of constructs containing two identical γ or β genes assessed the effect of gene order on globin gene expression, while analysis of constructs containing tandemly arranged γ and β genes assessed any additional effects of the *trans*-acting environment. When two γ genes were tandemly linked to the LCR, expression from the proximal γ gene was three- to fourfold higher than expression from the distal γ gene, and the ratio of proximal to distal gene expression remained unchanged throughout development. Similarly, when two β genes were tandemly linked to the LCR, the proximal β gene was predominantly expressed throughout development. These results indicate that proximity to LCR increases gene expression, perhaps by influencing the frequency of interaction between the LCR and globin gene promoters. An arrangement where the γ gene was proximal and the β gene distal to the LCR resulted in predominant γ-gene expression in the embryo. When the order was reversed and the γ gene was placed distally to the LCR, γ-gene expression in the embryo was still up to threefold higher than expression of the LCR-proximal β gene. These findings suggest that the embryonic *trans*-acting environment interacts preferentially with the γ genes irrespective of their order or proximity to the LCR. We conclude that promoter competition rather than gene order plays the major role in globin gene switching.

The human β-globin locus includes five active genes, ε, $^{G}\gamma$, $^{A}\gamma$, δ, and β, and a regulatory element termed the locus control region (LCR). This region is located 6 to 21 kb 5′ of the ε gene, and it contains a set of erythroid lineage-specific DNase I-hypersensitive sites that are present regardless of which globin genes are active (21, 23, 56). The LCR activates the β-globin locus (17, 19, 20, 23) and, in transgenic mice, provides high-level erythroid lineage-specific expression of erythroid or nonerythroid genes (6, 23, 44, 51). The level of expression of LCR-linked genes is determined by the copy number of the transgene, and it is independent of the chromosomal position in which the transgene is integrated (23).

The active genes of the β locus are arrayed in the order 5′-ε-$^{G}\gamma$-$^{A}\gamma$-δ-β-3′, an arrangement that also reflects the order in which these genes are expressed during development. The ε-globin gene is expressed in the erythrocytes derived from the blood islands of the yolk sac of the embryo. Subsequently, the site of hematopoiesis switches to the fetal liver, the ε gene is silenced, and the two γ-globin genes become active. Around birth, γ-globin gene expression ceases and is replaced by δ- and β-globin gene expression (49). The relationship between the 5′-to-3′ arrangement of globin genes and the order in which these genes are expressed during development is conserved in most mammals (11). Correlations between spatial arrangement of the genes of a locus and the temporal order in which these genes are expressed during development are also found in other multigene loci, such as the homeobox genes (36). The question is thus raised as to whether the spatial order of the genes of a locus determines the order of their expression during development. This question was experimentally examined in transgenic mice by Hanscombe et al. (25), who concluded that gene order is an important determinant of the developmental control of the β-like globin genes.

In the experiments reported here, we examined whether gene order or the presence of stage-specific *trans*-acting factors determines the temporal order of gene expression during development of transgenic mice. We first assessed the effect of gene order by analyzing expression of tandemly arranged γ or β genes in μLCRγγ or μLCRββ constructs. In these constructs, the transcription of proximal genes can be distinguished from the transcription of distal genes by RNase protection. Subsequently, we examined the relative roles of gene order and the *trans*-acting environment by analyzing expression of γ and β genes in transgenic mice carrying γβ- or βγ-gene constructs, i.e., constructs in which the γ gene is placed either proximally or distally to the LCR. Our results suggest that the primary determinant of appropriate gene expression during development is the *trans*-acting environment. Gene order (or distance from the LCR) influences the degree of gene expression, especially when two genes are structurally identical.

MATERIALS AND METHODS

General methods. Plasmid DNAs were maintained in or prepared from *Escherichia coli* DH5αMCR (gift from F. Bloom) to avoid rearrangements between cloned fragments. Transformation was performed by the method of Hanahan (24). Plasmid DNAs were prepared essentially by the alkaline lysis method (5) and then purified on pZ523 columns (5 Prime-3 Prime). Tail DNAs for slot blot analysis were prepared by a standard method (15). Probe DNAs were labeled by a modification of the random-primer method of Feinberg and Vogelstein (1, 45), using a Quick-Prime kit (Pharmacia).

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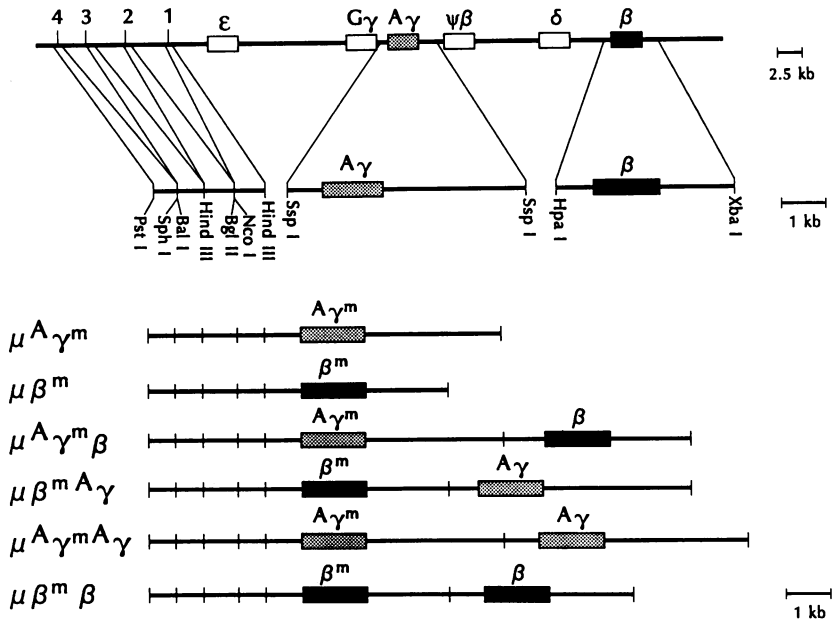

FIG. 1. Constructs used for testing the role of gene order, proximity to the LCR, or the *trans*-acting environment in the developmental control of β-globin genes. The μLCR fragment is the 2.5-kb *Eco*RI-*Hin*dIII cassette described by Forrester et al. (18). The 5.4-kb *Ssp*I γ-gene fragment and the 4.1-kb *Hpa*I-*Xba*I β fragment were derived from the human β-globin locus. The marked gene transcripts can be distinguished from those of the wild-type genes by RNase protection; γ^m is a 6-bp deletion (bp 39452 to 39457 [GenBank sequence]) in the 5′ untranslated region, and β^m is a 4-bp insertion (at bp 62239 [GenBank sequence]) in the 5′ untranslated region.

Constructs. A diagram illustrating the β-globin locus fragments used in creating the recombinant DNA constructs used in this study is shown in Fig. 1. Details of each construct are described below.

μLCRγβ. A 5.4-kb *Ssp*I fragment containing the human $^A\gamma$ gene was subcloned from p6.1JC1$^G\gamma^A\gamma$ (22) into *Ssp*I-*Sma*I-digested pUC19 (57). This plasmid, pUC19$^A\gamma$(+), was digested with *Apa*I and *Spe*I to remove 1.4 kb of the 5′ $^A\gamma$ flanking region and sequence; the vector plus the remaining $^A\gamma$ gene fragment was purified from an agarose gel by using DEAE-paper. A 1.4-kb *Apa*I-*Spe*I fragment containing the $^A\gamma$ marked ($^A\gamma^m$; a 6-bp deletion in the 5′ untranslated region from bp 39452 to 39457 [GenBank sequence]) was subcloned from pAγ* (43) into *Apa*I-*Spe*I-cut pUC19$^A\gamma$(+) to produce pUC19$^A\gamma^m$(+). pBSμHX2 was digested with *Eco*RI and *Hin*dIII, and the 5′ overhangs generated during the digestion were filled in with Klenow fragment to produce blunt ends. A 2.5-kb fragment containing the μLCR cassette (18) was isolated and subcloned into *Ssp*I-cut pUC19$^A\gamma^m$(+) to produce pμLCR(+)$^A\gamma^m$. pβBglII sense was digested with *Hpa*I and *Xba*I, the 5′ overhangs were filled in with Klenow fragment, and a 4.1-kb fragment containing the human β gene was purified and subcloned into *Sal*I-cut and filled pμLCR(+)$^A\gamma^m$ to produce pμLCR$^A\gamma^m\beta$.

μLCRβγ. pSP73β^m was digested with *Hpa*I and *Xba*I, and the resulting 5′ overhangs were filled in with Klenow fragment to produce blunt ends. A 4.1-kb fragment containing the marked β gene (β^m; the mark is a 4-bp insertion in the 5′ untranslated region at bp 62239 [GenBank sequence]) was purified and subcloned into *Hin*dIII-cut and filled pBSμHX2 to produce pμLCRβ^m(+). A 5.4-kb *Ssp*I $^A\gamma$ fragment was isolated from p6.1JC1$^G\gamma^A\gamma$ and subcloned into *Hin*dIII-cut and filled pμLCRβ^m(+) to produce pμLCR$\beta^{mA}\gamma$.

μLCRγγ. A 5.4-kb *Ssp*I fragment from p6.1JC1$^G\gamma^A\gamma$ encompassing $^A\gamma$ was subcloned into *Sal*I-cut and filled pμLCR(+)$^A\gamma^m$ to produce pμLCR$^A\gamma^{mA}\gamma$.

μLCRββ. A 4.1-kb *Hpa*I-*Xba*I fragment from pβBglII sense was filled in to produce blunt ends, purified, and subcloned into *Hin*dIII-cut and filled pμLCRβ^m(+) to produce pμLCR$\beta^m\beta$.

Transgenic mice. Fragments were purified by electroelution from agarose gels and used to inject eggs for the production of transgenic mice. All or most of the vector sequences were removed from the constructs by digestion with the proper restriction enzymes. Mice were examined for the presence of the injected fragment by slot blot analysis of tail DNA with 0.9-kb *Bam*HI-*Eco*RI human β-globin or 2.4-kb *Eco*RI human $^A\gamma$-globin fragments as probes. F_1 or F_2 embryos, fetuses, or adults were obtained from timed pregnancies at the days indicated in the figures and text.

RNase protection. Total cellular RNA was isolated from transgenic mouse tissues by the acid phenol method of Chomczynski and Sacchi (10). RNA concentration and integrity were assessed by UV spectrophotometry and agarose gel electrophoresis (1, 45). For RNase protection, 500 ng to 1 μg of yolk sac RNA, 500 ng of fetal liver RNA, or 200 ng of RNA from peripheral blood was hybridized overnight (15 to 18 h) at 45 to 50°C with 10^6 cpm of each radiolabeled probe. RNA probes were synthesized by in vitro transcription from linear templates, using either bacteriophage SP6 or T7 polymerase essentially as described previously (37). Template DNAs for riboprobe synthesis were pT7Mα, a derivative of pSP64 Mα (2) in which transcription of the probe is from the T7 promoter rather than the SP6 promoter, pT7β^m (15), pT7$^A\gamma^m$ (14), and pSP6Mζ (2). RNase protections were quantitated with a PhosphorImager (Molecular Dynamics). The level of $^A\gamma$, $^A\gamma^m$, β, or β^m human globin mRNAs was expressed as ratios of total human mRNA for a given construct in a particular sample.

Southern blot analysis. Genomic DNA was prepared from the carcasses of F_1 and F_2 progeny as previously described (15). Agarose gel electrophoresis, transfer of DNA to nylon

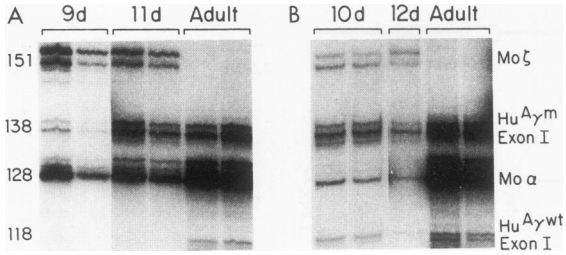

FIG. 2. RNase protection analysis of staged F_1 and F_2 progeny in μLCRγ^{m}γ transgenic mice. Total RNA (200 ng) from the blood of 9-, 10-, 11-, or 12-day fetuses and adults was hybridized with 10^6 cpm of RNA probes specific for human γ and β mRNAs or endogenous mouse α and ζ mRNAs overnight at 47°C (see Materials and Methods). The samples were digested with RNases A and T_1, and the protected radiolabeled fragments were subjected to electrophoresis on a sequencing gel. The human γ probe protects a different mRNA fragment for each of the two human ^{A}γ^m and ^{A}γ mRNAs, a 138-bp ^{A}γ^m exon I fragment (Hu ^{A}γ^m exon I), and a 118-bp ^{A}γ exon I fragment (Hu ^{A}γ^{wt} exon I [wt, wild type]). Mo ζ, 151-bp mouse ζ fragment; Mo α, 128-bp mouse α fragment. Radiolabeled pBR322 *Msp*I fragments were used as size markers, and RNAs isolated from known μLCRγ^m transgenic mice were used as controls in all RNase protections (data not shown). (A) Line B of Figure 3; (B) line C of Fig. 3. Sizes are indicated in base pairs.

membranes (Magnagraph NT; MSI, Inc.), and hybridization were performed by standard methods (1, 45). For copy number determination, 10 μg of genomic DNA was digested with *Eco*RI. After fractionation on agarose gels and DNA transfer, the blot was hybridized with a 2.4-kb *Eco*RI human 3′ Aγ-gene fragment probe and a 0.6-kb *Bam*HI mouse *lck* cDNA fragment probe derived from plasmid NT18 (gift from R. Perlmutter). The blot was subjected to PhosphorImager analysis, and the human transgene copy number was determined by comparison of the ^{A}γ band with the endogenous mouse *lck* band (diploid) after adjusting for the specific activity of the two probes.

RESULTS

The LCR-proximal gene of two tandemly linked human γ genes is preferentially expressed throughout development. The effects of gene order or distance from the LCR on globin gene expression have been analyzed before by using tandemly arranged γ and β or α and β genes (25). To control the variables introduced by having different promoters and genes normally expressed in different developmental stages, we produced μLCR γ-gene constructs containing two identical ^{A}γ genes. The γ gene used is contained on a 5.4-kb *Ssp*I fragment extending from −732 to +4662 bp relative to the γ cap site. The 5′ end of the γ gene contains all of the presently defined promoter motifs; the 3′ end contains the putative γ-gene enhancer (7). The transcript of the proximal gene of this construct can be distinguished from the transcript of the distal gene by the presence of a mark in the 5′ untranslated region in the proximal gene that results in a detectable difference in the two transcripts when they are analyzed by RNase protection (43). In a single copy of the transgene, the distance from the center of the core of 5′ DNase-hypersensitive site 2 (5′ HS2) of the μLCR to the TATA box of the proximal γ^m gene is 1.8 kb; the distance from 5′ HS2 to the distal γ TATA box is 7.3 kb. Two or more copies of the transgene tandemly repeated head to tail would still leave 6.0 kb between the distal γ TATA box and the μLCR 5′ HS2 of the second transgene copy. Head-to-head or tail-to-tail arrangements would not bring LCR sequences closer to the distal γ-gene copy. Thus, the proximal γ^m gene is always about three to four times closer to the μLCR than is the distal γ gene.

Using this construct, we produced four transgenic founders and established three lines. Yolk sac, liver, and blood samples were collected from F_1 and F_2 offspring of timed pregnancies of the three lines, and RNA prepared from these samples was subjected to RNase protection analysis. Data from RNA prepared from blood samples of two of these lines (lines B and C) are shown in Fig. 2. Endogenous embryonic mouse ζ mRNA and adult mouse α mRNA are present in 9- to 12-day fetuses; only mouse α mRNA is present in the samples from adults. The human γ gene is expressed throughout development. mRNA from the proximal γ^m gene is observed in all lanes, but mRNA from the distal γ gene is not detected in 9- and 11-day samples of line B and barely detected in the 10- and 12-day samples of line C. Distal γ-gene expression is clearly observed in the adult samples of both lines, but as in the fetus, expression is much less than that observed for proximal γ^m.

Quantitative data (Fig. 3) show that throughout development, the proximal γ gene is responsible for the majority of the total γ-gene expression. The ratio γ^m/γ^m + γ (where γ^m is the proximal gene of the pair) is about 0.8 for line A, 0.9 for line B, and 0.85 for line C at all stages of development.

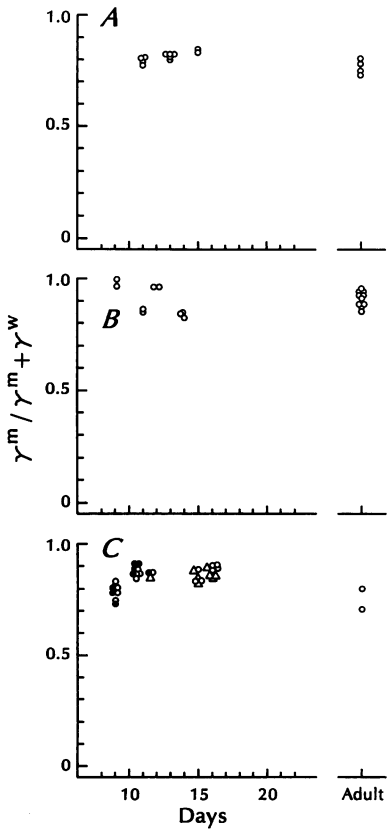

FIG. 3. Expression of the LCR-proximal γ^m gene predominates throughout development in μLCRγ^{m}γ transgenic mice. The level of γ^m mRNA was expressed as a percentage of total human γ mRNA (γ^m/γ^m + γ^w [w, wild type]) for individual samples. Symbols: ●, yolk sac; △, liver; ○, blood.

Since the two genes are structurally identical, the only factor that can be responsible for these differences is gene order or the distance between the genes and the LCR.

To examine whether the tandemly arranged γ genes are regulated during development, we calculated absolute expression of γ as a percentage of endogenous mouse α per gene copy. Previously, Enver et al. used μLCRγ constructs for similar studies and found a three- to fourfold decline in γ mRNA between the fetus and the adult (14, 15). Behringer et al. found a 5-fold decline with use of an LCR γ construct (4), while Dillon and Grosveld have reported 20- to 40-fold decreases in γ expression (13). In line A (with three copies of the transgene), γ expression per copy of transgene was 35% of α expression in 13-day fetuses, 45% of α expression in 15-day fetuses, and 2.2% of α expression in the adult. Thus, γ-gene expression displayed a 15- to 20-fold decline between the fetus and the adult. In line B, which has six copies of the transgene, the average γ expression (per copy of transgene) was 43% of α expression in the 12-day fetus, while it was 8.9% of α expression in the adult; thus, γ expression displayed about a fivefold decline during development. Line C had many (more than 100) copies of the transgene. Expression in F_1 fetuses ranged from 3.1 to 16% of α expression, while in the adults it ranged from 0.33 to 0.52% of α expression, indicating a 10- to 15-fold decline in γ-gene expression during development. Thus, both γ genes of the μLCR$\gamma^m\gamma$ construct are regulated during development; however, a switch from proximal to distal gene expression did not take place.

Analysis of β-gene expression in μLCR $\beta^m\beta$ transgenic mice. A construct linking two identical β genes in tandem array to the μLCR was used to test the effect of gene order or proximity to the LCR on β-globin gene expression. The β gene used is encompassed on a 4.1-kb *Hpa*I-*Xba*I β fragment extending from −817 to +3284 bp relative to the β cap site. This fragment contains all known 5′ regulatory sequences and the 3′ β-gene enhancer. The gene proximal to the μLCR contains an alteration in the 5′ untranslated region of the DNA encoding the mRNA, so that the mRNA products of the marked gene can be distinguished from those of the wild-type β gene by RNase protection (14). The distance from the core of 5′ HS2 in the μLCR to the TATA box of the proximal β^m gene is 1.9 kb; the same distance to the TATA box of the distal β gene is 6.1 kb. If the transgene is tandemly integrated head to tail two or more times, the μLCR of the second transgene copy would be 4.6 kb from the distal β gene of the first transgene copy. Head-to-head or tail-to-tail integrations would not juxtapose an LCR any closer to the distal β gene. Thus, in any configuration of multiple integrations, the proximal β^m gene will always be two to three times closer to LCR sequences than is the distal β gene.

Three founders were produced with the μLCR$\beta^m\beta$ construct, and two transgenic lines were established. RNase protection analysis was done with RNAs prepared from F_1 yolk sac and from liver and blood samples collected from the offspring of timed pregnancies of two μLCR$\beta^m\beta$ transgenic lines (corresponding to lines A and B in Fig. 4). One protected fragment is detected for the proximal β^m mRNA; two fragments are detected for the distal wild-type (β^{wt}) mRNA. The proximal β^m gene has a longer transcript due to the insertion used to create the mark (14). Thus, when β^m riboprobes are used, the mismatched sequences between β^m and β^{wt} hybrids are cleaved by the RNase to yield two β^{wt} fragments.

The RNase protection data of Fig. 4 shows that there is inappropriate expression of the β-globin gene in embryonic yolk sac cells (lanes containing RNA from 10- and 10.5-day yolk sacs), a phenomenon which has been previously documented in transgenic mice produced by using μLCRβ or LCRβ constructs (4, 16). It is also apparent that proximal β^m-gene expression is greater than distal β-gene expression at all stages of development.

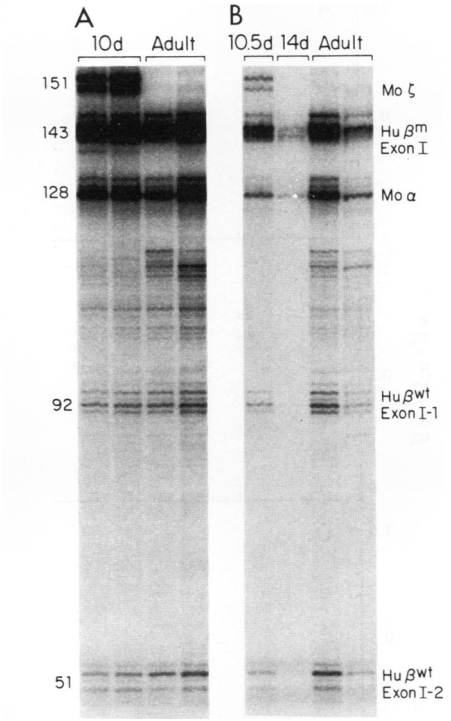

FIG. 4. RNase protection analysis of staged F_1 progeny in μLCR$\beta^m\beta$ transgenic mice. Total RNA (200 ng) from the blood of 9-, 10-, 10.5-, or 14-day fetuses and adults was subjected to RNase protection analysis. The human β probe protects three mRNA fragments from the two human β^m and β mRNAs, a 143-bp β^m exon I fragment (Hu β^m exon I), a 92-bp β exon I fragment (Hu β^{wt} exon I-1 [wt, wild type]), and a 51-bp β exon I fragment (Hu β^{wt} exon I-2). Mo ζ, 151-bp mouse ζ fragment; Mo α, 128-bp mouse α fragment. (A) Line A of Fig. 5; (B) line B of Fig. 5. Sizes are indicated in base pairs.

Quantitative data are shown in Fig. 5. From founder C (which had 43 copies of the μLCR $\beta^m\beta$ transgene), no transgenic F_1 progeny older than 9 days could be obtained. The mean $\beta^m/\beta^m + \beta$ ratio of the 9-day mice embryos was 0.55; in the founder, it was 0.6. In line A, the average $\beta^m/\beta^m + \beta$ ratio of the 10-day embryos was 0.83, while it was 0.81 in the adult animals. In line B, two embryonic 9 day samples gave a $\beta^m/\beta^m + \beta$ ratio of 0.5, but the measurements in the other stages of development provided a mean $\beta^m/\beta^m + \beta$ ratio of 0.68.

We conclude that gene order or proximity to the LCR influences β-gene expression, since the proximal gene was preferentially expressed in fetuses and in adults.

The γ-globin gene is preferentially expressed in embryos independently of gene order or proximity to the LCR. The results obtained with the μLCR$\gamma^m\gamma$ and μLCR$\beta^m\beta$ transgenic lines showed that proximity to the LCR (or gene order) affects gene expression when the genes are structurally identical. To assess the relative contributions of proximity to the LCR and the *trans*-acting environment on genes which are normally expressed at different development stages, we

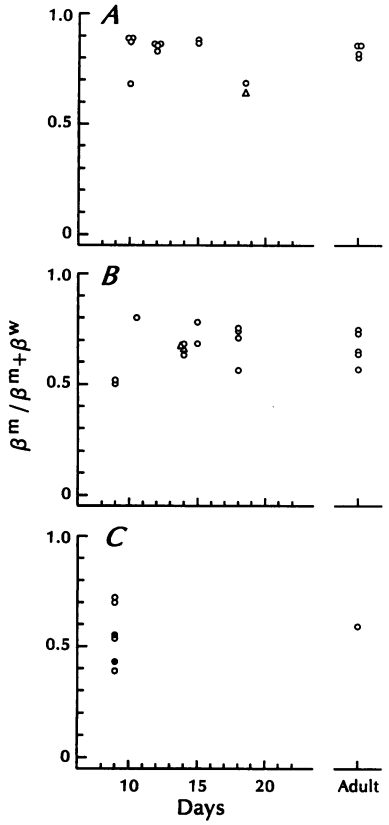

FIG. 5. The LCR-proximal β^m gene is predominantly expressed throughout development in μLCR$\beta^m\beta$ transgenic mice. The level of β^m mRNA was expressed as a ratio to total human β mRNA ($\beta^m/\beta^m + \beta^w$ [w, wild type]) for each sample. Symbols: ●, yolk sac; △, liver; ○, blood.

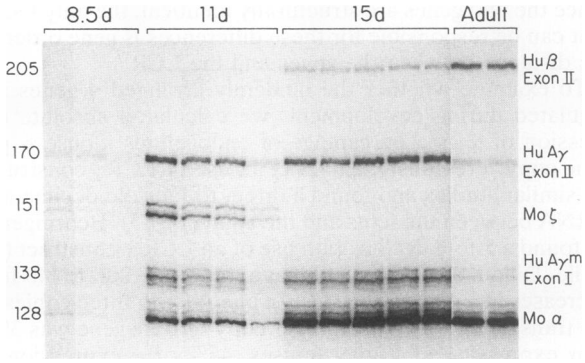

FIG. 6. RNase protection analysis of staged F_2 progeny in μLCR $\gamma^m\beta$ transgenic mice. Five hundred nanograms of total RNA isolated from the yolk sacs and carcasses of 8.5-day fetuses and 200 ng of total RNA isolated from the blood of 11- and 15-day fetuses or adults were analyzed. The human β probe protects one human β fragment, a 205-bp exon II fragment (Hu β exon II); the human γ probe protects two human $^A\gamma^m$ mRNA fragments, a 170 bp $^A\gamma$ exon II fragment (Hu $^A\gamma$ exon II) and a 138-bp $^A\gamma^m$ exon I fragment (Hu $^A\gamma^m$ exon I); the mouse α probe protects a 128-bp mouse α fragment (Mo α); and the mouse ζ probe protects a 151-bp mouse ζ fragment (Mo ζ). Sizes are indicated in base pairs.

produced transgenic mice carrying the μLCR$\gamma^m\beta$ and μLCR$\beta^m\gamma$ constructs. Hanscombe et al. (25) used similar arrangements of the γ and β genes to examine the role of gene order in developmental regulation and found that when the β-globin gene was in the LCR-proximal position, it was inappropriately expressed in the embryonic stage of development. In our μLCR $\beta\gamma$ construct, the distance from the core of 5′ HS2 of the μLCR to the β-globin gene promoter TATA box is about 2 kb; the distance from 5′ HS2 to the γ-gene promoter TATA box is about 6 kb. If the transgene is tandemly repeated head to tail, the core of 5′ HS2 of the second transgene copy μLCR will still be 6 kb from the distal γ-gene TATA box of the first transgene. Head-to-head or tail-to-tail arrangements will not place LCR sequences any closer to the γ gene.

The results of the developmental studies using the μLCR $\gamma^m\beta$ construct are shown in Fig. 6 and 7. γ mRNA accounted for almost all of the human globin mRNA in the 8.5-day fetuses and for 90% of human γ mRNA in 11-day fetuses. Subsequently, there was a decline in γ-gene expression, and in the adult animal the $\gamma/\gamma + \beta$ ratio was 0.28.

Three transgenic lines were produced by using the μLCR $\beta^m\gamma$ construct, and erythroid tissues from different developmental days were analyzed by RNase protection (Fig. 8). In line A, a strong human γ mRNA band and a weak β mRNA band can be seen in the 9-day embryo. In the 9-day embryos of lines B and C, γ-mRNA expression is higher than β-mRNA expression. γ mRNA is almost equal to β mRNA in the 11-day fetuses of lines A and B and less than β mRNA in the 12-day fetus of line C. There is only minor γ-gene expression in adult mice.

Quantitative data are presented in Fig. 9. In line A, the average $\gamma/\gamma + \beta$ ratio was 0.62 (range, 0.5 to 0.8) at day 9, 0.26 at day 12.5, 0.13 at day 15, and 0.05 in adults. A similar trend is observed for line B, in which the $\gamma/\gamma + \beta$ ratio was 0.74 at day 9, 0.52 at day 11.5, 0.38 at day 12, 0.11 at day 16, and 0.11 in adults. With only limited samples for line C, the data remain consistent, with a $\gamma/\gamma + \beta$ ratio of 0.75 in day 9 fetuses, 0.45 in day 11 fetuses, and 0.07 in adult transgenic mice.

These results show that high γ-gene expression is present in the embryo even when the normal order of γ and β genes is reversed and the γ gene is placed distally to the LCR.

DISCUSSION

Work with transgenic mice suggests that two mechanisms, an autonomous and a competitive mechanism, are involved in globin gene regulation. The autonomous mechanism is

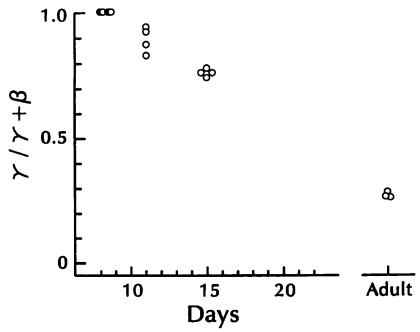

FIG. 7. Expression of human γ and β genes is developmentally regulated in μLCR$\gamma^m\beta$ transgenic mice. The level of $^A\gamma$ mRNA is expressed as a ratio to total human mRNA ($\gamma/\gamma + \beta$) for each sample. Symbols: ●, yolk sac and carcass; ○, blood.

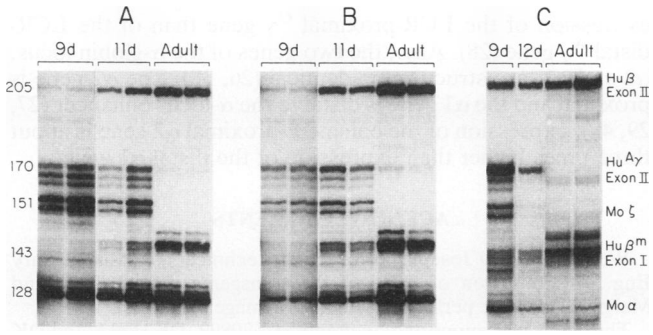

FIG. 8. RNase protection analysis of staged F_1 and F_2 progeny in μLCRβ^{m}γ transgenic mice. Total RNA (200 ng) from the blood of 9-, 11-, or 12-day fetuses and adults was subjected to RNase protection analysis. With this construct, the human β probe protects two human β^m mRNA fragments, a 205-bp exon II fragment (Hu β exon II) and a 143-bp exon I fragment (Hu β^m exon I). The human γ probe protects only the 170-bp ^{A}γ exon II fragment (Hu ^{A}γ exon II). Mo ζ, 151-bp mouse ζ protected fragment; Mo α, 128-bp mouse α protected fragment. Panels A, B, and C represent three different mouse lines.

illustrated by the results of the analysis of ε-globin gene expression in transgenic mice carrying μLCR ε (41) or 5′ HS2 ε (46) constructs, showing that ε-gene expression is normally restricted to embryonic erythropoiesis, but it continues in the adult stage of development when a silencer element upstream of the ε promoter is deleted (42). The competitive mechanism of switching is illustrated by the results obtained in transgenic mice carrying human γ- and β-globin genes.

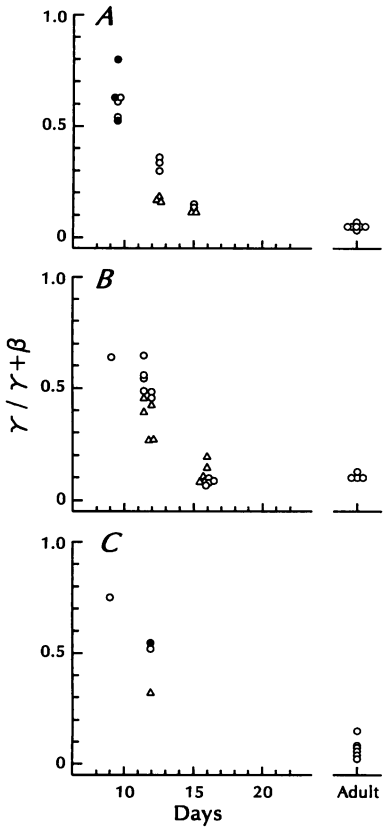

FIG. 9. Predominant expression of the distally placed γ gene in day 9 embryonic cells of transgenic mice. Human γ- and β-gene expression was quantitated as described in Materials and Methods. Symbols: ●, yolk sac; △, liver; ○, blood. Panels A, B, and C represent three separate mouse lines and correspond to panels A, B, and C in Fig. 8.

When transgenic mice are produced with γ-gene constructs lacking the LCR sequences, the γ gene behaves like its evolutionary orthologous gene, the mouse βh1 gene, and it is expressed only in embryonic cells (8, 12, 31). Other experiments have shown that β-gene expression is absent in the cells of embryonic erythropoiesis, but it is present in the cells of definitive erythropoiesis in transgenic mice carrying β-gene constructs lacking the LCR (12, 13, 30, 35, 54). These results suggested that the cells of the embryonic and definitive stages of erythropoiesis contain stage-specific transcriptional factors, while the γ and β genes contain the *cis*-acting sequences necessary to respond to these factors. Thus, globin gene switching could be explained by the changes in the *trans*-acting environment which occur during ontogeny (8, 12, 30, 35). Subsequent experiments, however, showed that developmental regulation of the γ- or β-globin genes is lost when these genes are linked to the LCR. Transgenic mice containing μLCRγ or LCRγ constructs expressed the γ gene in embryonic cells but also in the cells of definitive erythropoiesis of fetal liver and, at a lower level, in the cells of the adult bone marrow erythropoiesis (4, 15, 16, 34). When the β gene was linked to the LCR, it was inappropriately expressed in the embryonic cells of yolk sac erythropoiesis in addition to the cells of adult erythropoiesis (4, 16). Such results were interpreted to suggest that the LCR overrides the developmental regulation of γ- or β-globin genes even in the presence of the *trans*-acting factors which provide the developmental specificity of globin gene expression (16). Correct developmental regulation of γ and β genes was, however, restored when the two genes were present in cosmid constructs linked to the μLCR or to the LCR or to 5′ HS2 or coinjected with the LCR sequences (4, 16, 39, 50). The reappearance of developmental control in this experimental set up was explained (4, 16) by the hypothesis initially proposed by Choi and Engel (9) to account for globin gene switching in chickens. It was assumed that during the embryonic stage of development, the availability of embryonic stage-specific factors favors the interaction of the γ-globin gene with the LCR, while in the definitive stage of erythropoiesis, the presence of adult stage-specific factors favors the interaction between the LCR and the β-globin gene; as a result, the β gene is turned off competitively in the embryo while the γ gene is turned off competitively in the adult (4, 16, 40, 50, 52). Hanscombe et al. (25) subsequently proposed that, in addition to the *trans*-acting environment, gene order plays an important role in the developmental control of globin genes by increasing the probability of interaction between LCR and proximal globin genes. They proposed a model attempting to explain switching as the outcome of the combined effects of the *trans*-acting environment and the distances between the LCR and globin genes (25). Our experiment attempted to assess the importance of gene order or distance from the LCR in the control of globin gene expression. We approached this question by comparing the developmental expression of pairs of identical genes to the expression of pairs of nonidentical genes placed proximally or distally to the LCR.

When two β genes or two γ genes were linked to the LCR, both genes of a pair displayed identical expression during development. In the case of the two β genes, there was no developmental regulation and both genes were active in the

embryonic as well as in the adult (definitive) stages of erythropoiesis. The two γ genes behaved as the individual γ genes do in LCR γ-gene constructs; i.e., they displayed high expression in the fetus and low expression in the adult. With both constructs, expression of the LCR-proximal genes was higher than expression of the distal genes and the ratio of proximal to distal globin gene expression remained constant throughout development. The lack of a proximal-to-distal globin gene switch in these tandemly arranged genes excludes a simplistic model of switching whereby gene order per se, i.e., the 5′-to-3′ arrangement of the genes, plays a role in the switch of gene expression during development. In the μLCRγγ and the μLCRββ transgenic lines, expression of the proximal gene was two- to fivefold higher than expression of the distal gene. Since the proximal genes were about 4 to 6 kb closer to the LCR than were the distal genes, the most likely interpretation of the results is that the proximity to the LCR increases the probability of LCR-globin gene promoter interaction and consequently the level of expression of the proximal gene.

In view of the effect that gene order (or proximity to the LCR) has on the expression of the LCR-proximal genes, the results obtained with the construct in which the β gene was proximal and the γ gene was distal to the LCR are especially important. In this μLCRβγ construct, the β-gene promoter is 4 to 6 kb closer to the LCR than is the γ-gene promoter. Despite the distal location, expression of the γ gene in embryonic cells was two- to threefold higher than that of the proximal β gene. Thus, there was a higher chance for interaction between the LCR and the distal γ gene in the embryonic environment which favors this interaction. When the definitive stage of erythropoiesis began in the liver, there was a rapid γ-to-β-gene switch, as expected from the presence, in the fetal liver, of an adult *trans*-acting environment which favors β-gene expression. The higher γ/β ratio of globin gene expression in embryonic cells suggests that position is not an important determinant of developmental expression and that promoter competition plays the major role.

The role of gene order in the developmental control of globin genes can also be inferred from observations in vivo. Most animals display the correlation between 5′-to-3′ gene organization and 5′-to-3′ gene switching, but there are exceptions. The most notable exception is that of the genes of the β^A locus of the goat. This locus extends over 160 kb, and it has been formed through a triplication of a four-globin-gene set (53, 55). An LCR, which is structurally and functionally homologous to the human LCR, is found upstream of the first triplication unit (32, 33). The first gene to be expressed, in embryonic development, is the ε gene, which is also the first gene of the first triplication unit. During fetal development, the β^F gene, which is the last gene of the third triplication unit, becomes active. Around the perinatal period, there is a switch from β^F to β^C expression; the β^C gene is the fourth gene of the first triplication unit. Subsequently, there is a switch from β^C to β^A expression; the β^A gene is the fourth gene of the second triplication unit. Gene expression thus switches spirally from the 5′ to the 3′ end of the locus, again to the 5′ end of the locus, and finally to the middle of the locus, strongly arguing against the possibility that gene order plays a role in the control of developmental expression of the genes of the β locus.

Gene order, however, may modulate the level of expression of structurally identical globin genes. The $^G\gamma$- and $^A\gamma$-globin genes are structurally identical as a result of gene conversion (47). In the fetus, there is two- to threefold-higher expression of the LCR-proximal $^G\gamma$ gene than of the LCR-distal $^A\gamma$ gene (28). Also, the two genes of the α-globin locus, α2 and α1, are structurally identical (26, 38). The α2 gene is proximal and the α1 gene is distal to the α-locus enhancer (27, 29, 40); expression of the enhancer-proximal α2 gene is about three times higher than expression of the distal α1 gene.

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