MOLECULAR AND CELLULAR BIOLOGY, May 1992, p. 2057–2066
0270-7306/92/052057-10$02.00/0

Vol. 12, No. 5

A Single β-Globin Locus Control Region Element (5′ Hypersensitive Site 2) Is Sufficient for Developmental Regulation of Human Globin Genes in Transgenic Mice

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Received 4 October 1991/Accepted 4 February 1992

The β-globin gene complex is regulated by an upstream locus control region (LCR) which is responsible for high-level, position-independent, erythroid-cell-specific expression of the genes in the cluster. Its role in the developmental regulation of β-like globin gene transcription remains to be established. We have examined the effect of a single LCR element, hypersensitive site 2 (HS2), on the developmental regulation of the human fetal γ and adult β genes in transgenic mice. In mice bearing HS2^{A}γβ and HS2^{G}γ^{A}γ^{-117}δβ human globin gene constructs, switching from γ- to β-gene expression begins at about day 13.5 of gestation and is largely completed shortly after birth. The larger construct also demonstrates a switch in ^{G}γ- to ^{A}γ-gene expression during the γ-to-β switch similar to that observed during normal human development. We conclude that HS2 alone is sufficient for developmental regulation of the human β-globin genes.

The human β-globin gene cluster lies on the short arm of chromosome 11 and contains five genes arranged in the order of their developmental expression, 5′-ε-^{G}γ-^{A}γ-δ-β-3′ (37). The genes are under the control of a region at the 5′ end of the cluster known as the locus control region (LCR) (17, 20), which is marked by four DNase I-hypersensitive (HS) sites spread over approximately 20 kb (41). The LCR confers high-level, position-independent expression on attached genes in an erythroid-cell-specific manner in both transfected cell lines (4, 10, 16, 30, 38) and transgenic mice (3, 15, 20, 38). All four HS-site elements are necessary for maximum expression, but HS2 (10, 11, 16, 18, 30, 36, 40) and HS3 (16, 18, 32) are each highly effective alone in both experimental systems, while HS1 (18) and HS4 (18, 33) appear to augment the effects of these major activators. The role of the LCR in the developmental regulation of the cluster has yet to be determined.

Major determinants of developmental-stage-specific expression of the globin genes appear to reside in the area around the genes themselves. In transgenic mice, in the absence of the LCR, the human fetal γ genes are expressed at a low level at a time similar to that of mouse embryonic gene expression (6, 24), whereas the β gene alone is expressed in a developmental pattern similar to that of the endogenous mouse β gene (2, 24, 28). Attachment of the LCR, or of a smaller construct containing the four HSs (mini-LCR, or μLCR) or even HSs 1 and 2, to the β gene results in a loss of developmental regulation with high-level expression throughout development (3, 15). Results for γ-gene expression under control of the LCR elements have shown either expression throughout development but with a reduced level in later fetal and postnatal life (3, 14, 15) or restriction to the prenatal period (12). These inconsistencies between different studies may reflect either the exact nature of the construct used or the copy number of the exogenous genes in the transgenic mice (12). Restoration of developmental control to the β-gene cluster is observed when the LCR is coinjected with a 40-kb fragment containing the ^{G}γ^{A}γδ and β genes (3), with the μLCR attached to a fragment containing the ^{A}γδ and β genes in their normal chromosomal organizations (15), or with a μLCR ^{A}γβ construct (21); in these cases, only γ-gene expression is observed in the embryonic period, being completely replaced by β-gene expression later in development. These results suggest either that there is competition between the γ and β genes for expression throughout development (3, 15) or, alternatively, that the γ gene is autonomously regulated while the β gene is suppressed by competition from the γ gene in embryonic life (12, 21). A similar model has been proposed to explain hemoglobin switching in the chick (7).

The β LCR region has been highly conserved during mammalian evolution, with a similar organization of HSs in humans, mice (29), and goats (25); in goats, it has been shown that sequence conservation relative to that in humans extends considerably beyond the HSs themselves (26). However, the β-globin genes themselves have evolved separately in the three species in terms of their overall organization, number of active and inactive genes, and temporal pattern of expression. It is not clear, therefore, whether the arrangement of LCR sequences has been conserved for its function in activating the whole locus in erythroid cells or whether the sequence and relative positions of the HSs are involved in developmental control. HS2 and HS3 can each alone confer high-level expression on a linked globin gene in transgenic mice, but it is not yet clear whether either is capable of permitting developmental control if attached to both the fetal γ- and adult β-globin genes. We now show that HS2 alone will allow switching from human γ- to β-gene expression in transgenic mice and that the arrangement of the attached genes is important for their patterns of regulation.

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MATERIALS AND METHODS

Constructs. p N2γβ1 (referred to as HS2^{A}γβ) was constructed by inserting a 1.9-kb *Pvu*II-*Kpn*I fragment containing HS2 of the β-globin LCR into the *Apa*I site of p Nβ1.1, a plasmid containing the 4.9-kb *Bgl*II fragment of the β gene. A 3.2-kb *Hin*dIII-*Xmn*I fragment containing the ^{A}γ gene was inserted into the *Bgl*II site to give a 10-kb HS2^{A}γβ construct (30), which was released from the vector with *Not*I and gel purified prior to injection (see Fig. 1A).

We attempted to insert the same HS2 fragment into a cosmid containing the 39-kb *Kpn*I fragment, which spans the ^{G}γ^{A}γδ and β genes in their normal genomic orientations. This has so far been unsuccessful because of repeated rearrangements, but we have been able to insert this site into a similar cosmid which contains a G→A substitution at position −117 in the ^{A}γ gene, a mutation (9) which in vivo confers the phenotype of hereditary persistence of fetal hemoglobin (HPFH). The HS2 fragment was inserted into the unique *Sma*I site 5′ to the ^{G}γ gene (in the inverse orientation), and the whole human insert (referred to as HS2^{G}γ^{A}γ^{-117}δβ [30]) was released by digestion with *Asp* 718. Vector sequences were not removed prior to injection.

Transgenic mice. Transgenic mice were produced by microinjection of DNA into the male pronuclei of fertilized mouse eggs (CBA × C57B6), which were then transferred to pseudopregnant females. Developing progeny were either removed as 13- to 17-day fetuses or allowed to deliver naturally. Transgenic fetuses were screened by RNase protection analysis of peripheral blood or fetal liver samples, and mosaicism was checked by comparing the human-mouse DNA signals in samples of placenta, carcass, brain, and fetal liver. Live-born transgenic animals were identified by DNA analysis of a tail sample and were mated to B6/CBA F_1 mice to establish lines of transgenic mice. For studies of the developmental expression of the human globin genes in transgenic mice, male mice positive for the transgenes were mated to normal B6/CBA females; the morning on which a copulatory plug was observed was considered day 0.5 post coitus (p.c.).

DNA analysis. Screening for positive animals was carried out by digesting the DNA with *Eco*RI and using a human β IVS2 fragment (*Bam*HI-*Eco*RI fragment) as a probe. Positive animals were extensively analyzed by using the following additional probes: HS2 (*Bal*I-*Xba*I fragment), γ (*Pvu*II fragment), and ψβ (*Bgl*II-*Xba*I fragment). Tandem repeats were identified with *Bgl*II, *Hin*dIII, and *Bam*HI digests using the β IVS2 probe. Gene copy number was determined in first-generation progeny relative to a series of dilutions of the β IVS2 plasmid in normal mouse DNA calibrated against normal human DNA and using the mouse erythropoietin gene probe (*Pst*I fragment; kindly donated by P. Ratcliffe, University of Oxford) as an internal control for DNA concentration.

RNA analysis. RNA for globin gene expression studies was prepared from the following tissues: day 9.5 to 11.5 embryos, whole yolk sac, and embryo; day 12.5 to 18.5 fetuses, blood, and fetal liver; postnatal animals, blood, spleen, and bone marrow. In order to determine tissue specificity of expression, RNA was isolated from the following additional tissues from one adult animal of each line; brain, muscle, heart, lung, liver, kidney, thymus, intestine, and testis or ovary.

RNA was prepared by the method of Chomczynski and Sacchi (8) and was analyzed by the quantitative RNase protection assay of Zinn et al. (44). The following probes were used: (i) for mouse α, pSPJMαS, a 103-bp *Pst*I-*Bal*I fragment linearized with *Hin*dIII to give a protected fragment of 93 bp (R. W. Jones, University of Oxford); (ii) for human β, pGβ1, a 174-bp *Bst*NI fragment linearized with *Eco*RI to give a 135-bp protected fragment (30); (iii) for human 5′ γ probe hybridizing with both ^{G}γ and ^{A}γ transcripts, pG^{G}γ2.1, a 212-bp *Bst*EII-*Msp*I fragment linearized with *Eco*RI to give a 142-bp fragment (30); (iv) for human 3′ γ, pBA^{A}γ, a 516-bp *Hin*dII-*Rsa*I fragment linearized with *Hin*dII and giving protected fragments of 215 bp with ^{A}γ RNA and 131 bp with ^{G}γ RNA (M. Harvey, University of Sydney); (v) for mouse ζ, pSP64Mζ, an *Ava*II-*Xba*I fragment linearized with *Eco*RI and producing a 151-bp protected fragment (1).

RNA (0.1 to 2.0 μg) was hybridized overnight with 10^6 cpm of up to four different [α-^{32}P]GTP-labeled probes, always including mouse α as an internal control for endogenous globin gene expression. After digestion with RNases A (40 μg/ml) and T1 (2 μg/ml), the protected fragments were separated on 8% acrylamide–8 M urea gels and autoradiographed. Controls with a two- to threefold increase in the amount of RNA hybridized were carried out regularly to ensure that samples were analyzed in probe excess.

Quantitation of the RNA was performed by excising the bands from the gel and counting them in the presence of scintillant. The radioactive counts were corrected for the number of C residues in the protected fragments: α^M = 19, ζ^M = 48, β^H = 34, 5′ γ = 31, and 3′ γ = 54 in the ^{A}γ band and 36 in the ^{G}γ band. For more-accurate quantitation of RNAs present at low amounts (i.e., β in early embryos or γ in postnatal samples), 10- to 20-fold more RNA was hybridized separately with the probe for the less-abundant RNA than was hybridized with the α^M probe and the probe for the

TABLE 1. Copy numbers and expression data for HS2^{A}γβ embryos

Embryo	Gestational age (days)	Copy no.	% γ + β/α^M RNA/copy[a]	% β/β + γ RNA in:	
				Blood	Liver
B2	14	2	26	27	48
D1	18	8[b]	54	69	81
F3	19	3	84	63	76
G1	19	6	62	83	89

[a] Assuming that two α genes are equivalent to one β gene.
[b] Copy number analysis of various tissues from this fetus suggested that it was a mosaic. The copy number used is that obtained from the fetal liver.

TABLE 2. Copy numbers and expression data for transgenic mice

Line	Copy no.	% γ + β/α^M RNA[a]	
		Total	Per copy[b]
HS2^{A}γβ			
M2	3	42	28
T1	5	43	17
HS2^{G}γ^{A}γ^{-117}δβ			
J14	2	35	35
J17	3	45	30
J19	10	2	0.4
J21	8	27	7

[a] Values given are the means obtained from two to six adult mice from the first or second generation.
[b] Assuming that two α genes are equivalent to one β gene.

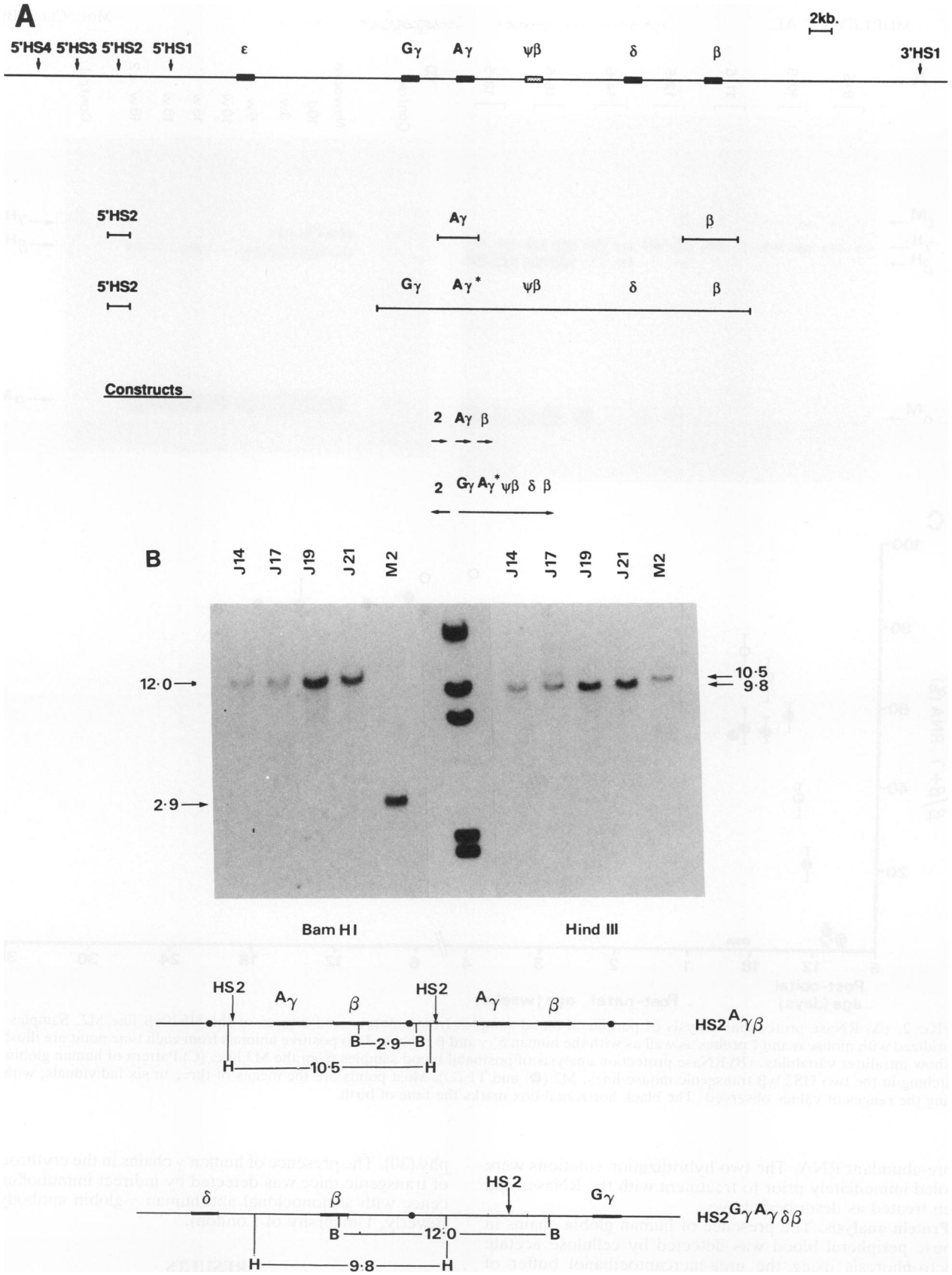

FIG. 1. (A) Organization of the constructs used in this study. Note that the 5′ HS2 fragment is in the inverse orientation in the $HS2^{G}\gamma^{A}\gamma\delta\beta$ construct; previous studies have demonstrated that the LCR and the HS2 fragments function equally well in either orientation (30, 38). (B) Transgenic-mouse-progeny DNAs digested with *Bam*HI and *Hin*dIII and hybridized with the human β-globin gene probe. The major bands correspond in size to those expected for head-to-tail tandem repeats. Minor bands presumably are due to junction fragments. Maps of the *Bam*HI (B) and *Hin*dIII (H) sites at the boundaries of tandem repeats are shown below the autoradiograph.

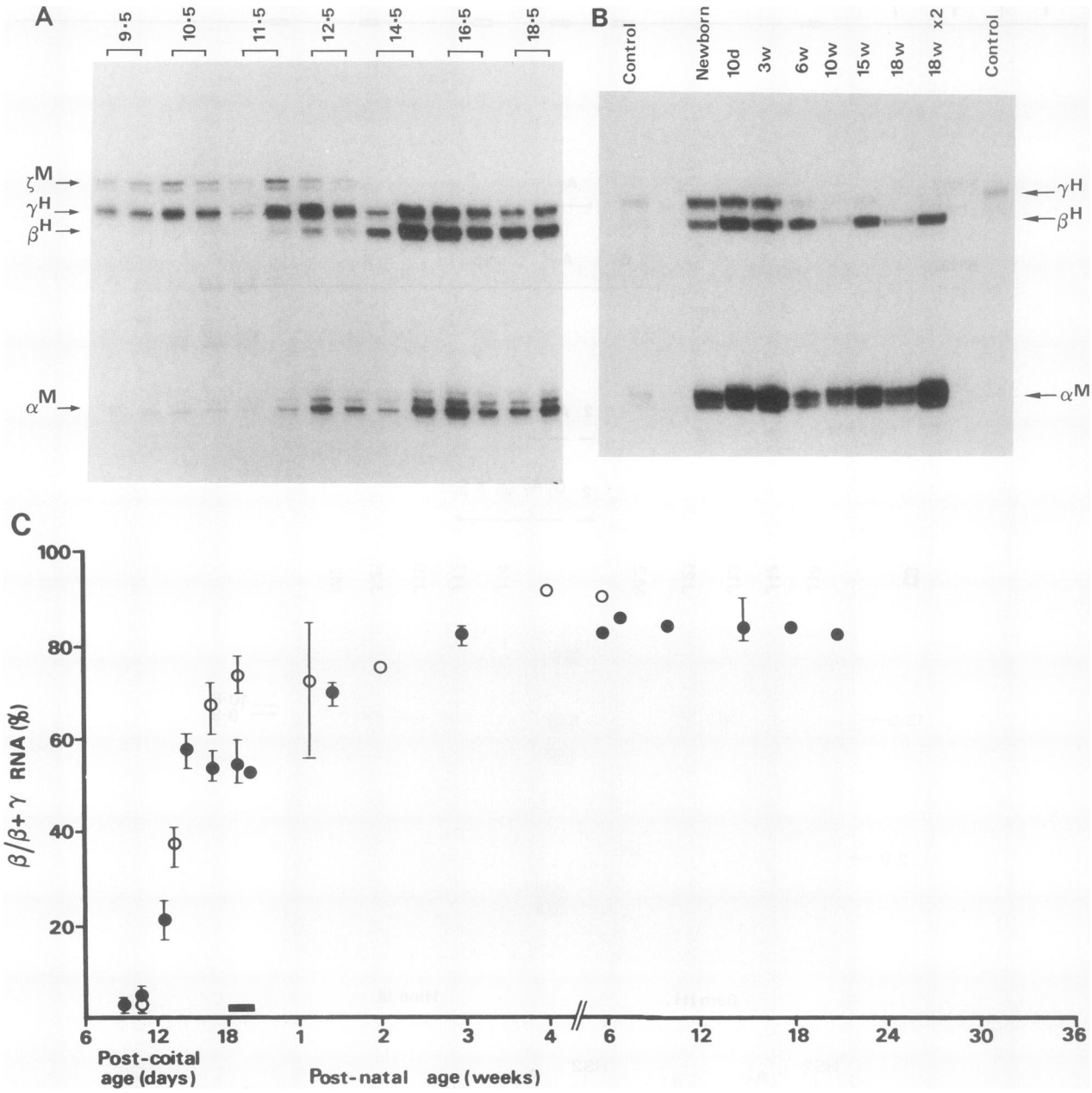

FIG. 2. (A) RNase protection analysis of peripheral-blood samples from embryos and fetuses of the HS2^{A}γβ line M2. Samples were hybridized with mouse α and ζ probes as well as with the human 5′ γ and β probes. Two positive animals from each time point are illustrated to show intralitter variability. (B) RNase protection analysis of postnatal blood samples from the M2 line. (C) Pattern of human globin gene switching in the two HS2^{A}γβ transgenic mouse lines, M2 (●) and T1 (○). Most points are the means of three to six individuals, with bars giving the ranges of values observed. The black horizontal box marks the time of birth.

more-abundant RNA. The two hybridization solutions were pooled immediately prior to treatment with the RNases and then treated as described above.

Protein analysis. The presence of human globin chains in mouse peripheral blood was detected by cellulose acetate electrophoresis using the urea-mercaptoethanol buffer of Huisman and Jonxis (23). Globin chain synthesis in peripheral blood, bone marrow, fetal liver, and spleen samples was carried out by incubating the cells with [^{3}H]leucine and measuring incorporation of the label into individual globin chains separated by carboxy methyl cellulose chromatography (30). The presence of human γ chains in the erythrocytes of transgenic mice was detected by indirect immunofluorescence with a monoclonal anti-human γ-globin antibody (P. Beverly, University of London).

RESULTS

Of the mice injected with the HS2^{A}γβ fragment, four embryos expressing the gene and two newborn mice which went on to establish transgenic lines were obtained. Results obtained from the individual embryos are summarized in

Table 1. Four mice positive for the $HS2^{G}\gamma^{A}\gamma^{-117}\delta\beta$ construct were obtained. Two of these gave rise to lines; one female consistently failed to rear her litters, and one failed to transmit the gene to any of 27 progeny (Table 2).

The number of copies of the inserted fragments ranged from 2 to 10 (Tables 1 and 2). In all cases, the inserted DNA remained unrearranged (data not shown), and when digests designed to detect tandem arrays of the inserts were performed, a prominent band in the position expected for head-to-tail repeats was observed (Fig. 1B) for each line. The average expression level of the human genes relative to the mouse α genes in each line is also given in Tables 1 and 2. Among adult animals, the ratio of human/mouse globin mRNAs is reasonably constant (27 to 45%) except for the nontransmitting line J19, despite variability in copy number. Indeed, there is an inverse correlation between copy number and mRNA output per copy of the human genes. Such a relationship has been noted previously in MEL cells (22, 30). In transgenic mice, both the intact LCR and HS2 constructs have been reported to give copy number-dependent levels of gene expression except at very high copy numbers (18, 20, 38, 39). Others, however, have observed a poor correlation between copy number and expression levels (5, 11, 16, 36). At present, the reasons for these discrepant results remain uncertain.

The output of total human globin RNA relative to α^{M} RNA was not always constant during development. At times earlier than day 12.5 of gestation, α^{M} RNA production was not maximal, but even after this, the ratio relative to that seen in adult animals was higher during prenatal life in three lines (M2, T1, J17) and lower in one line (J14). Such variability has also been observed previously but in transgenic mice with globin gene inserts lacking an LCR element (28). In all of the lines, human globin gene expression was erythroid cell specific (data not shown).

Hemoglobin switching in $HS2^{A}\gamma\beta$ lines. The pattern of human γ- and β-globin RNA production during development in the lines containing $HS2^{A}\gamma\beta$ is illustrated in Fig. 2. The pattern of endogenous mouse embryonic (ζ^{M}) and adult α genes is shown in Fig. 2A. At the earliest stages of gestation examined, 9.5 days p.c., both human genes are expressed, but γ accounts for approximately 95% of the total human RNA present. During fetal life, the proportion of β mRNA increases, reaching a semiplateau of around 50 to 70% between 14.5 and 20.5 days p.c. (Fig. 2A). The level continues to rise slowly during the weanling stage, reaching levels of ~80%. At each stage during development, RNA extracted from the fetal liver showed a higher proportion of β to β+γ mRNA than did peripheral-blood RNA, which is consistent with fetal liver erythroblasts being a later cohort of cells than those in the peripheral blood. Among adult animals, the proportion of β mRNA was rather variable, ranging from 70 to 90%, but in every case, γ mRNA remained easily detectable (Fig. 2B). The patterns of hemoglobin switching were similar in both lines (Fig. 2C), and the results in the four independent animals obtained as fetuses were generally consistent with the results in the two lines. Thus, while developmental regulation is observed with $HS2^{A}\gamma\beta$, both genes are expressed at all stages of development and the switching process is relatively gradual.

Globin chain synthesis (Fig. 3) in erythroid tissues from adult mice containing $HS2^{A}\gamma\beta$ showed levels of β^{H} chain synthesis of 12 to 27% relative to mouse α or β chains after a 60-min incubation, in contrast to β^{H} mRNA levels of ~40%. This discrepancy is likely to be due to posttranslational loss of the human β chains, since a time course study

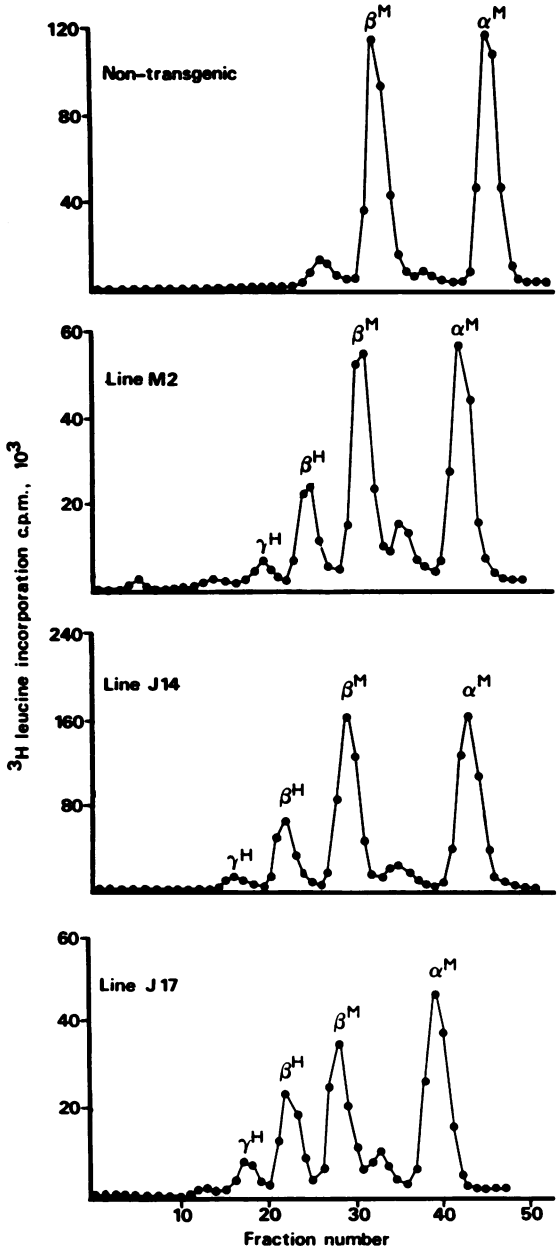

FIG. 3. Globin chain synthesis in adult mice of the M2 ($HS2^{A}\gamma\beta$), J17, and J14 ($HS2^{G}\gamma^{A}\gamma^{-117}\delta\beta$) transgenic lines and in a nontransgenic control. Globin chains are always preceded by "pre-peaks"; it is assumed that part of the human β-chain peaks consists of pre-β mouse chains, while the peak corresponding to the human γ chain is likely to contain mostly pre-β human chains. For the purposes of calculating the relative proportions of human and mouse chains, the ratios of pre-β^{M}/β^{M} and pre-α^{M}/α^{M} averaged from eight nontransgenic mice were used as a correction factor.

showed β^{H}-chain synthesis levels of up to 40% of β^{M}-chain synthesis levels at short incubation times (5 min); these levels decreased as the incubation was prolonged. On cellulose acetate electrophoresis of globin chains, the amount of human β chains was low, just at the level of detection.

Little or no γ-chain synthesis could be detected in adult mice containing the $HS2^{A}\gamma\beta$ construct, but the presence of γ chains in the erythrocytes was readily demonstrable by

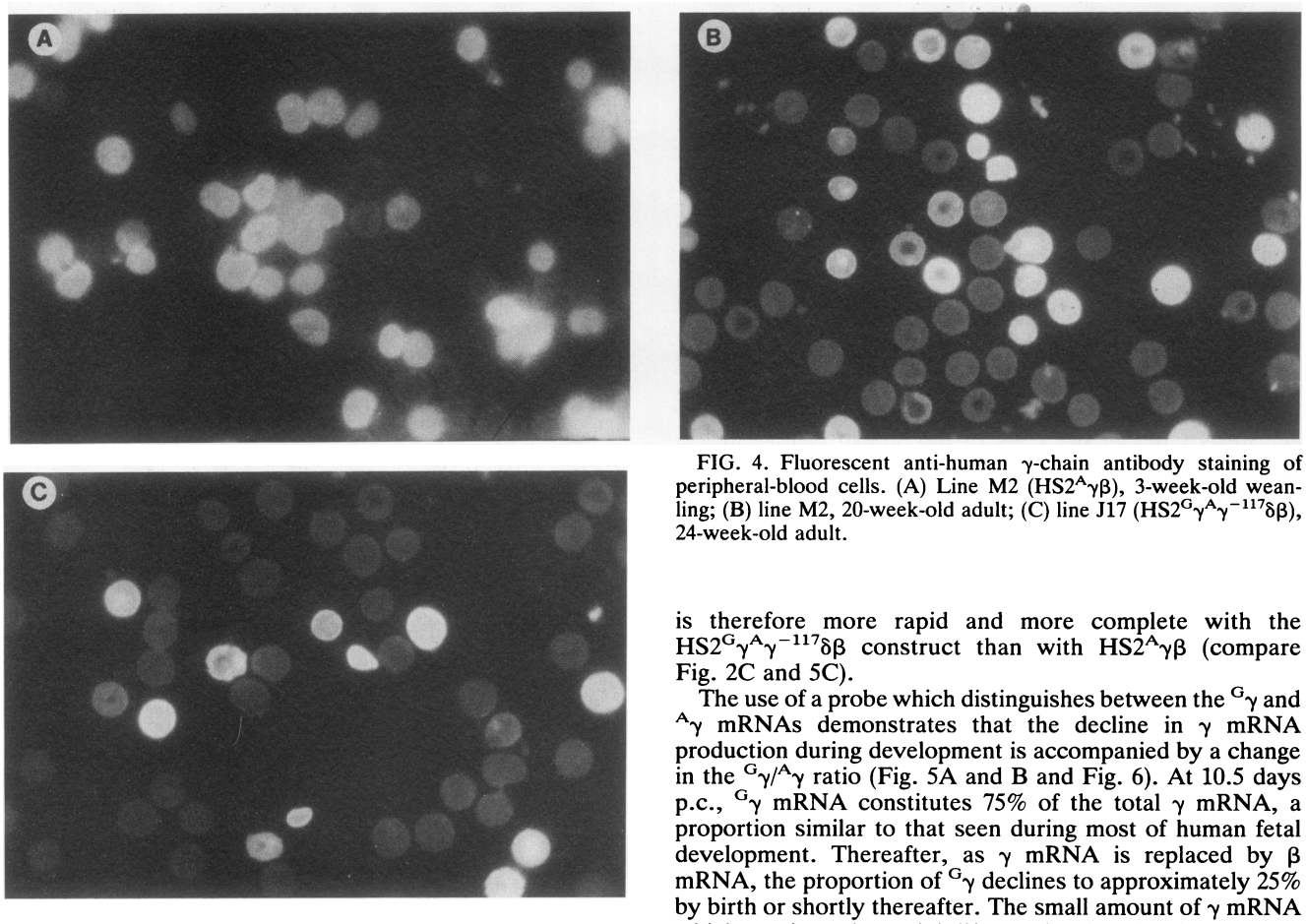

FIG. 4. Fluorescent anti-human γ-chain antibody staining of peripheral-blood cells. (A) Line M2 ($HS2^{A}\gamma\beta$), 3-week-old weanling; (B) line M2, 20-week-old adult; (C) line J17 ($HS2^{G}\gamma^{A}\gamma^{-117}\delta\beta$), 24-week-old adult.

immunofluorescence. Newborn animals contained detectable γ chains in all their erythrocytes but with considerable intercellular variability. By 7 weeks after birth, only 27 to 40% of cells were positive for γ chains by immunofluorescence. This value declined further, to 10 to 25%, by 6 months (Fig. 4).

Morphological inspection of peripheral blood smears, fetal liver cells, and adult spleen and bone marrow cells showed no overt abnormalities by light microscopy. Furthermore, in matings between hemizygotes for the transgenic and normal mice, the transgene was transmitted to 56 of 108 and 37 of 75 offspring in the two lines, with no evidence of a decreasing proportion in fetuses versus those examined post weaning, suggesting that in the hemizygous state, the presence of up to five copies of the transgene was not deleterious. However, matings between hemizygotes for the M2 line produced 7 nontransgenic animals and 17 hemizygotes but no homozygotes among those surviving to weaning. The stage at which homozygotes are lost has yet to be determined.

Hemoglobin switching in $HS2^{G}\gamma^{A}\gamma^{-117}\delta\beta$ lines. The pattern of human globin gene switching in the two $HS2^{G}\gamma^{A}\gamma^{-117}\delta\beta$ lines is shown in Fig. 5. At the earliest times, 9.5 to 12.5 days p.c., only γ mRNA was observed (Fig. 5A and B); no β^{H} mRNA was detectable even when 10-fold more total RNA was hybridized with the β probe. β mRNA became detectable at 13.5 days p.c. and then increased as a proportion of the total human globin RNA during the remainder of gestation, reaching its adult values of ~95 to 97% shortly after birth (Fig. 5A and B). Switching is therefore more rapid and more complete with the $HS2^{G}\gamma^{A}\gamma^{-117}\delta\beta$ construct than with $HS2^{A}\gamma\beta$ (compare Fig. 2C and 5C).

The use of a probe which distinguishes between the $^{G}\gamma$ and $^{A}\gamma$ mRNAs demonstrates that the decline in γ mRNA production during development is accompanied by a change in the $^{G}\gamma/^{A}\gamma$ ratio (Fig. 5A and B and Fig. 6). At 10.5 days p.c., $^{G}\gamma$ mRNA constitutes 75% of the total γ mRNA, a proportion similar to that seen during most of human fetal development. Thereafter, as γ mRNA is replaced by β mRNA, the proportion of $^{G}\gamma$ declines to approximately 25% by birth or shortly thereafter. The small amount of γ mRNA which continues into adult life consists largely, therefore, of $^{A}\gamma$ RNA.

Globin chain synthesis (Fig. 3) in adults bearing the $HS2^{G}\gamma^{A}\gamma^{-117}\delta\beta$ insert demonstrated detectable human β-globin chain synthesis at levels of ~ 25 to 40% of mouse β-chain production in the J17 line and ~5 to 18% in the J14 line, again lower than the relative proportions of the mRNAs (45 and 35%, respectively). Time course studies again showed selective degradation of the human β chain in the J17 line, with the ratio of β^{H}/β^{M} decreasing with increasing incubation time, from 41% after 5 min to 26% after 1 h.

Human β-globin chains were readily detectable in hemolysates from the J17 line; lower levels were observed in the J14 line. Immunofluorescence analysis of the γ-chain distribution in mice bearing the $HS2^{G}\gamma^{A}\gamma^{-117}\delta\beta$ construct showed a pancellular but heterogeneous distribution at birth, while in adult samples, the proportion of γ-positive cells ranged from 15 to 44% (Fig. 4).

Hemizygotes for the transgene showed no obvious hematological abnormalities; peripheral-blood smears showed normal erythrocyte morphology, bone marrow was normocellular with a normal myeloid/erythroid ratio, and there was no sign of extramedullary hemopoiesis in the spleens or livers of adult animals. Breeding data from lines J14 and J17 showed 31 of 75 and 39 of 80 transgenic offspring, respectively, from hemizygous × normal-mouse matings. Interestingly, in line J14, with lower copy number and expression levels, 20 of 42 offspring examined at less than 15 days of gestation were transgenic, while only 11 transgenic animals were obtained in 33 offspring older than this. Equivalent figures for the J17 line were less discrepant, i.e., 16 of 31 and

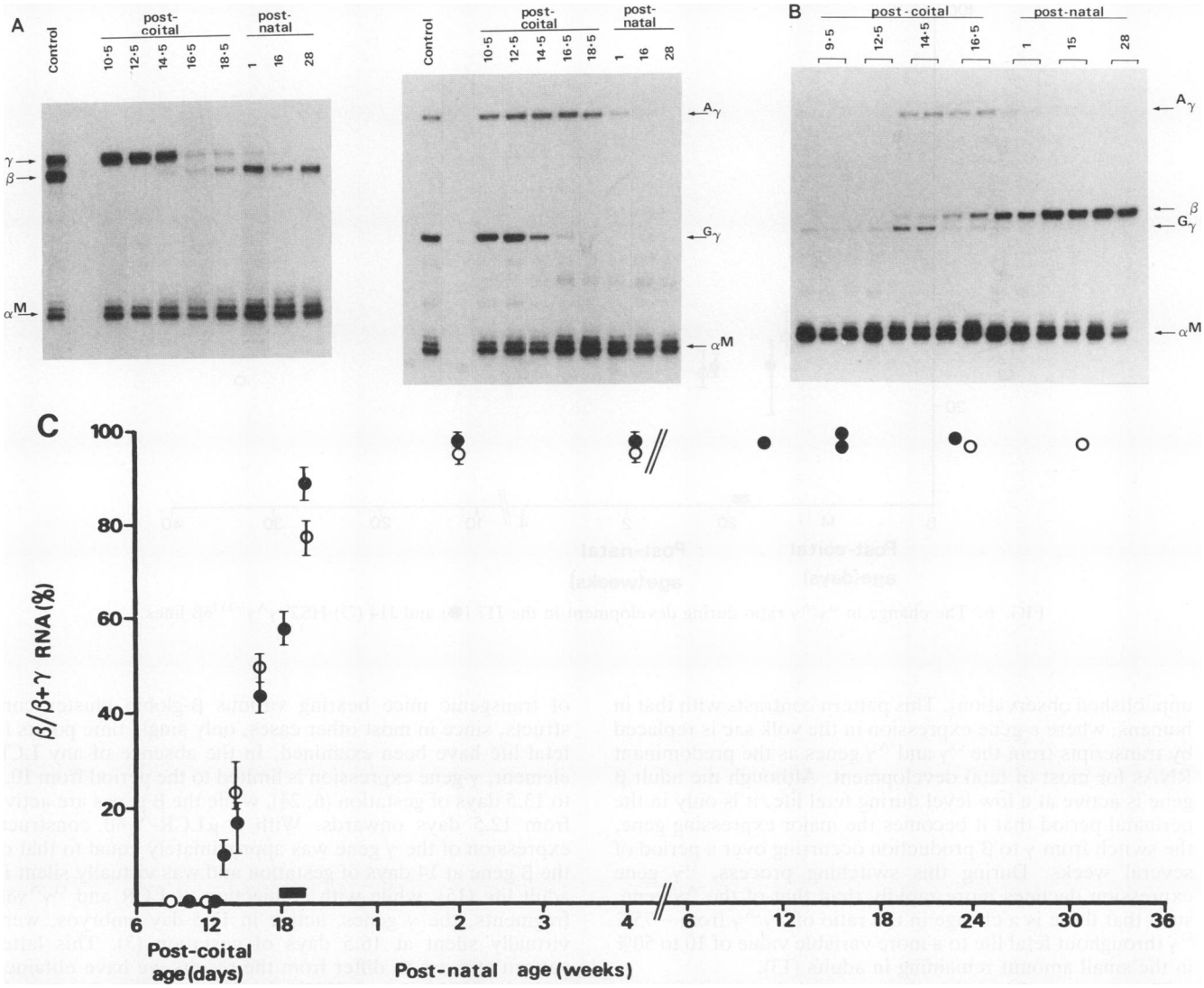

FIG. 5. (A) RNase protection assay of peripheral-blood samples from individuals of the HS2$^{G}\gamma^{A}\gamma^{-117}\delta\beta$ line J17 at various stages (age given in days) of development. On the left, the samples were hybridized with human 5′ γ and β probes to show the switch in the total γ/β ratio while on the right, the same samples were hybridized with the 3′ γ probe to demonstrate the switch in $^{G}\gamma/^{A}\gamma$ ratio. The control samples are a mixture of human cord and adult reticulocyte RNAs with induced MEL-cell RNA. Different exposures of the two gels were used to equalize the amounts of globin RNA hybridized. (B) RNase protection assay of peripheral-blood samples from individuals of the HS2$^{G}\gamma^{A}\gamma^{-117}\delta\beta$ line J14 hybridized with human β and 3′ γ probes and with the mouse α probe (age given in days). (C) Overall pattern of human globin gene switching in the two HS2$^{G}\gamma^{A}\gamma^{-117}\delta\beta$ lines, J17 (●) and J14 (○).

23 of 49, respectively. From matings of two hemizygotes, 1 homozygote was produced in 24 offspring in the J14 line and 1 of 20 was produced in the J17 line. In each case, the homozygous animal was considerably smaller than the normal or hemizygous littermates; their hematology has not yet been examined.

DISCUSSION

The data presented here demonstrate for the first time that a single HS site from the β-globin LCR is sufficient to allow switching from human γ- to β-gene transcription in transgenic mice. Previous studies have demonstrated that LCR elements attached to a β gene alone promote expression of the gene throughout development in transgenic mice (3, 15), the effect of the LCR elements overriding the developmental regulation seen with the β gene in the absence of any LCR elements (24, 28). In contrast, when both γ and β genes are attached to HS2, either as the small 10-kb fragment HS2$^{A}\gamma\beta$ or with the 45-kb HS2$^{G}\gamma^{A}\gamma^{-117}\delta\beta$ construct, developmental regulation is restored. These results, therefore, are generally similar to those obtained when intact LCR or μLCR sequences are used with γ-β constructs (3, 15, 21).

During normal mouse development, the embryonic hemoglobins produced during the yolk sac phase of erythropoiesis are replaced directly by adult hemoglobins once the fetal liver becomes the major site of erythropoiesis. Within the mouse β-gene cluster, there is asynchrony in the expression of the two embryonic genes, with βhl predominating prior to day 11.5 of gestation, when εy^{2} becomes the major form (42). However, both genes are silent by day 15 of gestation (our

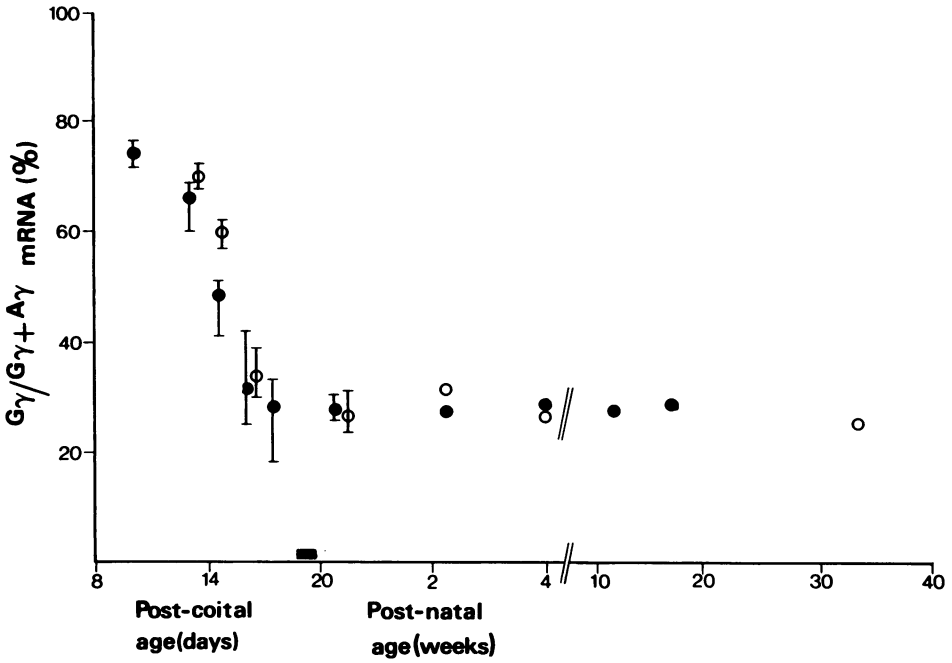

FIG. 6. The change in $^{G}\gamma/^{A}\gamma$ ratio during development in the J17 (●) and J14 (○) HS2$^{G}\gamma^{A}\gamma^{-117}\delta\beta$ lines.

unpublished observation). This pattern contrasts with that in humans, where ε-gene expression in the yolk sac is replaced by transcripts from the $^{G}\gamma$ and $^{A}\gamma$ genes as the predominant RNAs for most of fetal development. Although the adult β gene is active at a low level during fetal life, it is only in the perinatal period that it becomes the major expressing gene, the switch from γ to β production occurring over a period of several weeks. During this switching process, $^{G}\gamma$ gene expression declines more rapidly than that of the $^{A}\gamma$ gene, such that there is a change in the ratio of $^{G}\gamma/^{A}\gamma$ from ~75% $^{G}\gamma$ throughout fetal life to a more variable value of 10 to 50% in the small amount remaining in adults (13).

The pattern of human γ-gene expression in the transgenic mice described here does not correspond to that of either of the endogenous mouse embryonic genes, with γ RNA persisting throughout in utero development. In the HS2$^{G}\gamma^{A}\gamma^{-117}\delta\beta$ transgenic lines, there is a switch in the $^{G}\gamma/^{A}\gamma$ ratio, which again does not correspond to the time of the change in the mouse embryonic β globin but rather occurs later, at the same time as the switch from γ- to β-gene expression, just as it does during normal human development. It is not clear whether these temporal changes in $^{G}\gamma$-, $^{A}\gamma$-, and β-gene expression reflect alterations (qualitative or quantitative) to the complement of *trans*-acting factors in mouse erythroid cells, since they occur after the endogenous genes have reached their stable adult patterns of expression. As yet, no developmental-stage-specific *trans*-acting factors have been isolated from mammalian erythroid cells, although they do apparently occur in the chick (19). A clearer understanding of the *cis*-active sequences which confer developmental regulation on the human β-globin genes, their possible modification during development (30), and their interactions with transcription factors within the mouse erythroid cells will be needed before the timing of the switching process can be understood at the molecular level.

It is not clear whether the pattern of hemoglobin switching in these mice matches completely those in previous studies of transgenic mice bearing various β-globin cluster constructs, since in most other cases, only single time points in fetal life have been examined. In the absence of any LCR element, γ-gene expression is limited to the period from 10.5 to 13.5 days of gestation (6, 24), while the β genes are active from 12.5 days onwards. With a μLCR-$^{A}\gamma\delta\beta$ construct, expression of the γ gene was approximately equal to that of the β gene at 14 days of gestation and was virtually silent in adult life (15), while with coinjection of LCR and $^{G}\gamma^{A}\gamma\delta\beta$ fragments, the γ genes, active in 11.5-day embryos, were virtually silent at 16.5 days of gestation (3). This latter pattern appears to differ from the results we have obtained with the HS2$^{A}\gamma\beta$ and HS2$^{G}\gamma^{A}\gamma^{-117}\delta\beta$ transgenic mice, in which the switch from γ- to β-gene expression largely occurred between day 13.5 of gestation and 1 week postnatally. Until the pattern of hemoglobin switching in transgenic mice bearing a single copy of the whole human β-globin complex is known, it will not be clear to what degree the smaller constructs used here and in previous studies deviate from this pattern.

Although the patterns of globin gene switching with the two constructs are broadly similar (Fig. 2C and 5C), there are differences, principally that the process is more rapid and more complete in the HS2$^{G}\gamma^{A}\gamma^{-117}\delta\beta$ transgenic lines. This tighter control occurs despite the fact that the $^{A}\gamma$ gene in this construct contains a mutation which results in vivo in an HPFH phenotype and the production of about 10% hemoglobin F in heterozygotes (9). It remains to be seen whether the small residual levels of $^{A}\gamma$ mRNA in adult animals bearing this fragment are a result of this mutation. Nevertheless, the consistent differences between the HS2$^{A}\gamma\beta$ and HS2$^{G}\gamma^{A}\gamma^{-117}\delta\beta$ lines suggests that the precise pattern of hemoglobin switching does depend on the structural arrangement of the genes, although it is not clear whether this dependence is on their spatial organization or on sequences between the genes themselves. In this regard, it has been suggested that the enhancer sequence (34) 3′ to

the $^{A}\gamma$ gene may play a role in the suppression of γ-gene expression (12, 27). This sequence is absent from our HS2$^{A}\gamma\beta$ construct, as it is from the LCR$^{A}\gamma$ (3) and μLAR$^{G}\gamma$ (15) constructs of others, all of which produce significant levels of γ-gene expression in adult transgenic animals.

The overall structure of the LCR is similar in humans (20), mice (29), and goats (25). Not only are the HS sites maintained, but their relative spacing and positions with respect to the globin genes are similar in all three species. Furthermore, extensive sequencing of the goat LCR (26) has demonstrated that high-level sequence homology is maintained over a considerable distance and is not restricted to the areas containing the HS sites. Such a high level of sequence conservation normally implies an important functional role for such sequences. The fact that a single HS site is sufficient not only to allow high-level expression of attached genes but also to allow developmental control raises questions about the redundancy observed in the β LCR. It is possible that an intact LCR is necessary to provide fine tuning of regulation, affecting either the level of gene expression or its temporal control or both.

The developmental regulation of human globin gene expression from those constructs in transgenic mice contrasts with that observed (30) from the same constructs after transfection into MEL cells, an "adult" erythroid cell line. Equal expression of both $^{A}\gamma$ and β genes was observed from the HS2$^{A}\gamma\beta$ construct, while the ratio of $^{G}\gamma/^{A}\gamma/\beta$ RNAs from the larger construct was 1:2:1 in MEL cells. When human genes are introduced into MEL cells on an intact chromosome, developmental regulation is observed (31, 35, 43). Clearly, the present results demonstrate that the structures of these constructs do not prevent their developmental regulation. The expression of all genes in transfected MEL cells, despite the cells' essentially adult phenotype, implies that developmental selection of gene expression does not rely solely on a change in the *trans*-acting factor complement of erythroid cells. Rather, it suggests that modification of the globin sequences occurs at some stage during their development in transgenic mice. By using the lines described here, this possibility can be tested, and such modifications can now be sought.

ACKNOWLEDGMENTS

We thank M. Sampietro for the immunofluorescence studies and N. Roberts for technical assistance. We are grateful to D. J. Weatherall for his support and to D. R. Higgs for his advice and encouragement.

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