Human γ- to β-globin gene switching in transgenic mice

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Previous studies demonstrated correct tissue- and temporal-specific expression of human γ- and β-globin genes in transgenic mice; however, expression was extremely low. When the erythroid-specific DNase I super-hypersensitive (HS) sites that are normally located upstream of the human β-globin locus were fused individually to γ- or β-globin genes, expression increased to endogenous mouse globin levels but temporal specificity was lost. In contrast, when the HS sequences were combined with fragments containing both γ- and β-globin genes, correct developmental regulation was restored. We suggest that human γ- to β-globin gene switching during development results from competition of individual globin gene family members for interaction with the HS sequences and that factors influencing these competitive interactions determine temporal specificity.

[*Key Words*: DNase I super-hypersensitive sites; locus activation region; globin gene switching; temporal specificity; transgenic mice]

Received October 23, 1989; revised version accepted December 12, 1989.

The human β-like globin genes reside on the short arm of chromosome 11 in a cluster of five active genes (5′-ε-^{G}γ-^{A}γ-δ-β-3′), occupying ~50 kb of contiguous DNA (Fig. 1A). These genes are expressed specifically in erythroid tissue at high levels and are differentially expressed in a precise temporal manner during development in coordination with the α-like globin genes. The ε-globin genes are expressed during early embryonic development in erythroid cells located in the blood islands of the yolk sac. As development proceeds, ε synthesis ceases and the fetal γ-globin genes become activated when the major site of hematopoiesis switches to the fetal liver. Later in gestation, another switch occurs in which expression of the γ genes is replaced by adult δ- and β-globin genes, primarily in the erythroid cells of the bone marrow. Although these globin gene switches represent one of the classic examples of developmental control in eukaryotes, little is known about the mechanisms that mediate them.

When the human β-globin gene is assayed for activity in transgenic mice, expression is found specifically in adult erythroid tissue in a temporal pattern paralleling that of the endogenous mouse β-globin genes (Costantini et al. 1985; Townes et al. 1985b; Kollias et al. 1986). Human γ-globin genes are also expressed in an erythroid-specific manner with expression that parallels the expression of the endogenous mouse embryonic genes that are active during embryonic stages and inactive in adult erythroid tissue (Chada et al. 1986; Kollias et al. 1986). These results suggest that *cis*-acting regulatory elements that confer tissue- and temporal-specific expression are closely associated with the human β- and γ-globin genes. Although expression of human globin genes in transgenic mice is tissue-specific and temporally correct, the level of expression of each transgene is low compared to that of endogenous mouse β-globin genes. These results suggest that other sequences are required for correct quantitative regulation.

The human β-like globin gene cluster is flanked by multiple DNase I super-hypersensitive (HS) sites that are present specifically in erythroid cells at all developmental stages (Tuan et al. 1985; Forrester et al. 1986, 1987). The location of these sites defines the active β-like globin gene domain in cells of the erythroid lineage, and the sites appear to be required for activation of the locus. Support for this idea comes from studies of patients possessing deletions within the human β-globin locus in which the β-globin gene remains intact but some or all of the HS sites are missing (Bunn and Forget 1986; Driscoll et al. 1989). A characteristic phenotype of these patients is β-thalassemia, suggesting that the HS sequences normally act in *cis* to activate β-globin gene expression. The functional activity of HS sites was tested directly by ligating them to human α- and β-globin genes and assaying for expression in transgenic mice. High-level (Grosveld et al. 1987; Ryan et al. 1989a,b;

Talbot et al. 1989), erythroid-specific (Behringer et al. 1989; Ryan et al. 1989a,b) expression of the linked globin genes was attained in all mice at levels related to transgene copy number. Similar results were obtained in murine erythroleukemia cells in culture (Blom van Assendelft et al. 1989; Forrester et al. 1989; Talbot et al. 1989). Subsequent deletion analysis of the HS site region demonstrated that high-level expression of a linked β-globin gene could be achieved in mice with constructs containing only a single HS site (HS II) (Ryan et al. 1989b). These studies demonstrate that the HS sites are critical for high-level, erythroid-specific expression of human β-globin genes and suggest that proper regulation of the entire human β-globin locus requires the presence of the HS sites.

As described above, expression of human ε-, γ-, and β-globin genes occurs at defined stages during human development. This precise regulation requires that the powerful enhancing effects of the HS sequences must be precisely controlled. To study the effects of HS sequences on temporal specificity, we inserted human γ- and β-globin genes separately downstream of the HS sequences and assayed their expression at various developmental stages.

Results

HS β-globin genes are active during embryonic development

We have established three transgenic mouse lines that contain erythroid-specific DNase I HS sites inserted upstream of both the human α- and β-globin genes (Behringer et al. 1989, and unpubl.) and a line containing the human β-globin gene alone (Townes et al. 1985b; Fig. 1B). Transgenic mice were mated to normal animals, and progeny were removed at 11 or 16 days of development or were allowed to develop into adults. Embryos, fetuses, and adults that contained the transgenes were identified by DNA dot hybridization, and total RNA was examined for correctly initiated human and mouse globin mRNA by primer extension with five different oligonucleotides. Figure 2A illustrates the pattern of globin gene expression in 11-day embryos. Lane 1 is

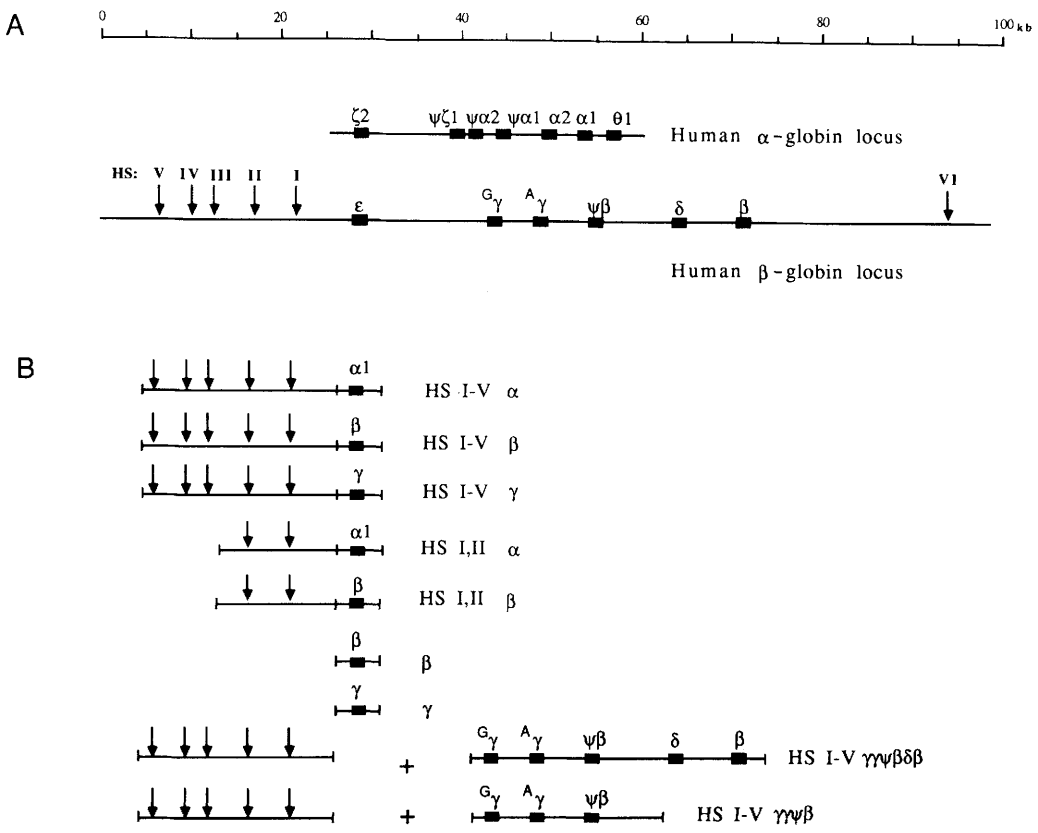

Figure 1. DNA fragments from the human α- and β-globin loci injected into fertilized mouse eggs. (*A*) Thirty-five kilobases of the human α-globin locus and 100 kb of the human β-globin locus are illustrated. Globin genes and pseudogenes are represented by black boxes. Locations of the DNase I HS sites are shown by arrows and designated by Roman numerals I–VI. (*B*) Human globin gene constructs injected into fertilized mouse eggs. DNA fragments containing individual globin genes (β or γ), globin genes fused to HS sites I and II (HS I,II α or HS I,II β), or globin genes fused to HS sites I–V (HS I–V α, HS I–V β, or HS I–V γ) were purified from vector sequences and injected individually or in various combinations into fertilized mouse eggs. HS sites I–V were coinjected with either a 27.5-kb fragment containing ^{G}γ-, ^{A}γ-, and ψβ-globin genes (HS I–V γγψβ) or a 40-kb fragment containing ^{G}γ-, ^{A}γ-, ψβ-, δ- and β-globin genes (HS I–V γγψββδβ) into fertilized mouse eggs.

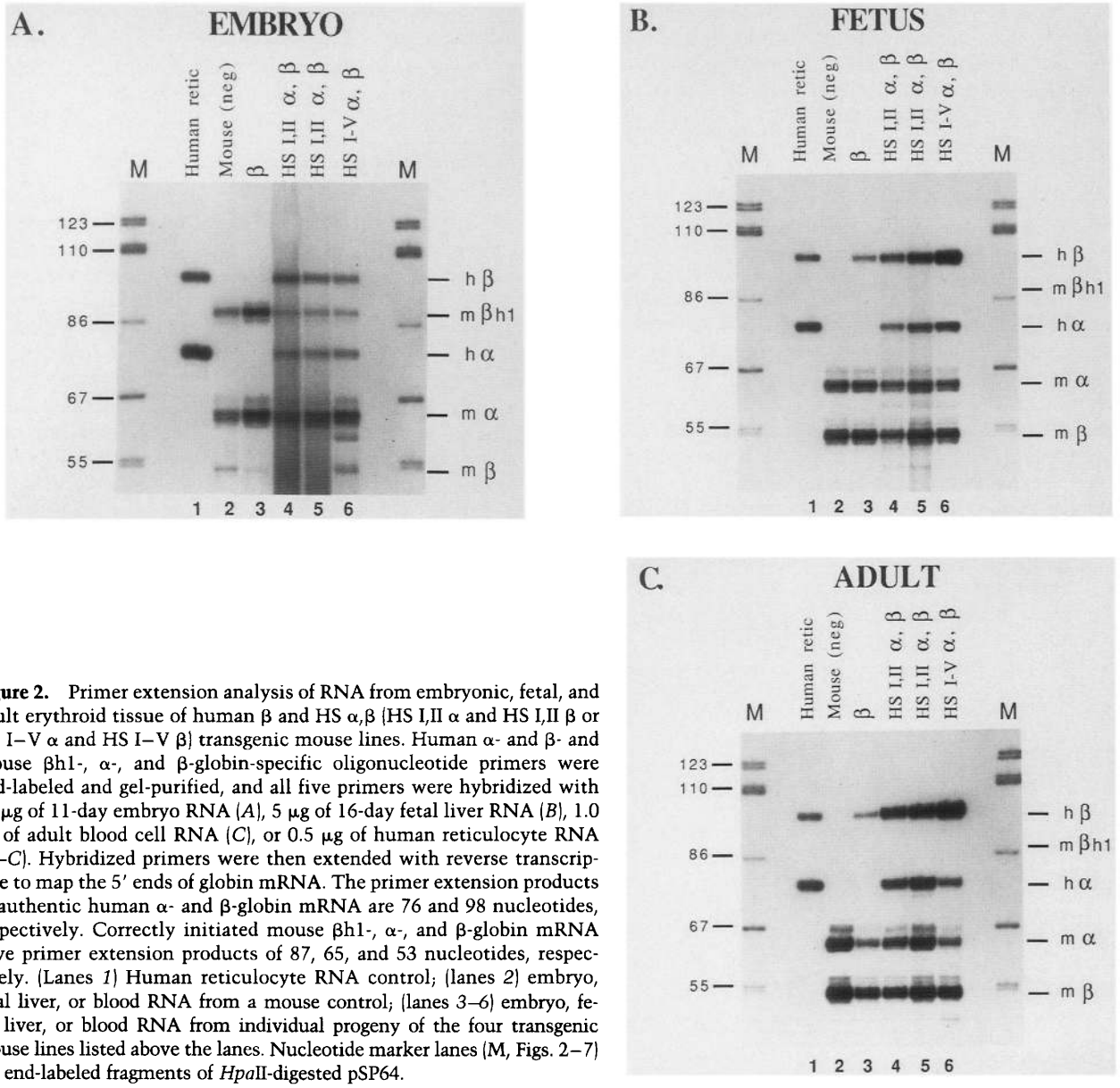

Figure 2. Primer extension analysis of RNA from embryonic, fetal, and adult erythroid tissue of human β and HS α,β (HS I,II α and HS I,II β or HS I–V α and HS I–V β) transgenic mouse lines. Human α- and β- and mouse βh1-, α-, and β-globin-specific oligonucleotide primers were end-labeled and gel-purified, and all five primers were hybridized with 20 μg of 11-day embryo RNA (*A*), 5 μg of 16-day fetal liver RNA (*B*), 1.0 μg of adult blood cell RNA (*C*), or 0.5 μg of human reticulocyte RNA (*A–C*). Hybridized primers were then extended with reverse transcriptase to map the 5′ ends of globin mRNA. The primer extension products of authentic human α- and β-globin mRNA are 76 and 98 nucleotides, respectively. Correctly initiated mouse βh1-, α-, and β-globin mRNA have primer extension products of 87, 65, and 53 nucleotides, respectively. (Lanes *1*) Human reticulocyte RNA control; (lanes *2*) embryo, fetal liver, or blood RNA from a mouse control; (lanes *3–6*) embryo, fetal liver, or blood RNA from individual progeny of the four transgenic mouse lines listed above the lanes. Nucleotide marker lanes (M, Figs. 2–7) are end-labeled fragments of *Hpa*II-digested pSP64.

human reticulocyte RNA, and lane 2 is an 11-day mouse control. These two lanes demonstrate the primer extension products of authentic human α- and β-globin mRNA and mouse α-, β-, and βh1-globin mRNA. Lane 3 is total RNA from an 11-day transgenic mouse embryo carrying 20 copies of the human β-globin gene without HS sequences. Although high levels of mouse α- and βh1-globin mRNA are observed, no human β-globin mRNA is detected. This result is consistent with our previous data from this line, demonstrating that like the endogenous mouse β-globin genes, human β-globin transgenes are not expressed in early mouse embryos (Townes et al. 1985b). The HS β transgenes, on the other hand, are expressed at high levels in 11-day embryos, as illustrated in lanes 4–6. High-level human β-globin gene expression is observed for embryos containing constructs with only HS sites I and II (lanes 4 and 5) and for embryos containing a construct with sites I–V (lane 6). These results demonstrate that correct temporal specificity is lost when the human β-globin gene is inserted immediately downstream of the HS sequences.

The pattern of mouse and human globin gene expression is illustrated in 16-day fetal liver (Fig. 2B) and adult blood (Fig. 2C) from the same transgenic lines. Mice switch directly from embryonic (βh1) to adult β-globin gene expression when the major site of erythropoiesis shifts from the yolk sac to the fetal liver at ~13 days of gestation; β-globin expression then persists throughout life (cf. Fig. 2A–C, lanes 2). Figure 2, B and C, lanes 3, demonstrates that the human β-globin gene was activated concomitantly with the endogenous mouse β-globin genes in fetal liver and adult blood in the line containing the human β-globin gene alone (Townes et al. 1985b). In the lines containing HS β-globin transgenes,

expression that was established at a high level in the 11-day embryo persisted at a high level throughout fetal and adult development.

HS γ-globin genes are expressed in adult erythroid tissue

We then analyzed expression of the human γ-globin gene inserted downstream of the HS sequences. Chada et al. (1986) and Kollias et al. (1986) demonstrated that the human γ-globin gene was expressed in erythroid cells of the yolk sac during early embryonic development and was subsequently turned off in fetal liver and bone marrow. Fertilized eggs were injected with HS I–V $^{A}\gamma$, or the $^{A}\gamma$ gene alone (Fig. 1B), and implanted into the oviducts of foster mothers. After 16 days of gestation, the fetuses were removed and total fetal liver RNA was analyzed for γ-globin mRNA by primer extension. Figure 3 illustrates the results of this experiment. Lane 1 is human fetal liver RNA, and lane 2 is fetal liver RNA from a mouse control. Lanes 3–5 are fetal liver RNA from three animals that contain the $^{A}\gamma$-globin gene alone. As expected, no γ-globin mRNA was detected. All five animals that contained the HS I–V $^{A}\gamma$ construct, however, expressed correctly initiated human γ-globin mRNA (lanes 6–10). The average level of γ expression was 26% of endogenous mouse β-globin per gene copy. These results demonstrate that like the β-globin gene, correct temporal-specific expression of the human $^{A}\gamma$-globin gene in transgenic mice is lost when the gene is inserted immediately downstream of the HS sequences.

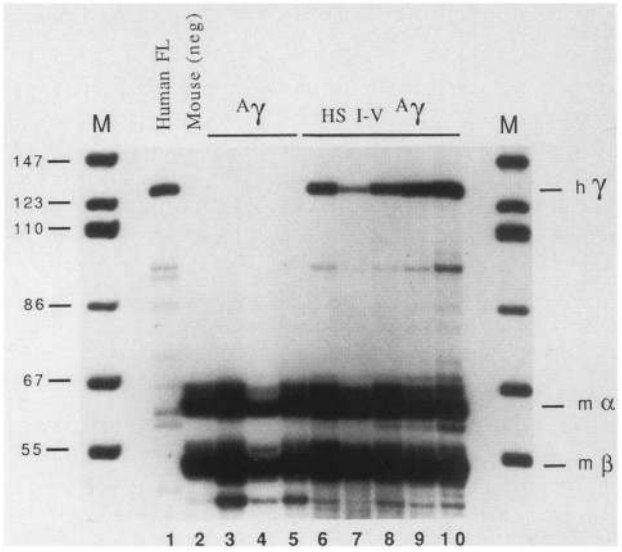

Figure 3. Primer extension analysis of 16-day fetal liver RNA from human γ and HS I–V γ transgenic mice. Human γ-, mouse α-, and β-globin-specific oligonucleotide primers were end-labeled, gel-purified, hybridized to 16-day fetal liver RNA, and extended with reverse transcriptase. (Lane *1*) 1.0 μg of human fetal liver RNA control; (lane *2*) 5 μg of fetal liver RNA from a mouse control; (lanes *3–5*) 5 μg of fetal liver RNA from transgenic fetuses containing the human $^{A}\gamma$-globin gene alone; (lanes *6–10*) fetal liver RNA from transgenic fetuses containing the HS I–V γ construct. The primer extension product of authentic human γ-globin mRNA is 127 nucleotides.

We also established lines of mice carrying HS γ-globin transgenes to analyze expression throughout development. Fertilized eggs were injected with equal molar amounts of HS I–V γ, HS I–V β, and HS I–V α constructs (Fig. 1B). Injected eggs were implanted into the oviducts of foster mothers. Two founder animals were generated that possessed intact copies of all three transgenes. These transgenes were transmitted to progeny as a single unit, suggesting that they had integrated together at a single chromosomal location. Southern blots of tail DNA demonstrated that the transgenes were arranged in a head-to-tail tandem array. These mice were mated to control animals, and progeny were removed at 11 or 16 days of development or were allowed to develop into adults. Total RNA was then examined for correctly initiated human and mouse globin mRNA by primer extension with six different oligonucleotide primers. Figure 4, A and B, illustrates the temporal pattern of expression in transgenic progeny. The first four lanes are the following controls: human reticulocyte RNA (lane 1), human fetal liver RNA (lane 2), mouse 11-day embryo RNA (lane 3), and mouse 16-day fetal liver RNA (lane 4). The last three lanes are RNA from transgenic 11-day embryos, 16-day fetal livers, and adult blood, respectively. In both transgenic lines, the human α-, β-, and γ-globin genes were expressed at all stages of development. In 11-day embryos, human γ-globin expression was equivalent to the mouse embryonic gene (βh1). The relative level of human γ-globin mRNA decreased in fetal liver and blood but still remained high. Human γ- to mouse β-globin mRNA levels in these two lines averaged 42% per gene copy in fetal liver and 16% per gene copy in adult blood. On the other hand, the ratio of human β- to mouse β-globin mRNA increased during the development of 11-day embryos to adults. Therefore, although the human transgenes appear to respond partially to temporal signals, the HS sequences apparently override these controls and direct expression at all developmental stages.

Proper temporal regulation of human γ- and β-globin genes in adult and embryonic erythroid tissue of HS I–V γγψβδβ transgenic mice

The results described above demonstrate that HS sequences disrupt normal temporal-specific expression when they are inserted immediately upstream of the human γ- or β-globin gene. Presumably, the HS sequences dominate proximal regulatory sequences within and surrounding these genes and inhibit globin gene switching. How are these dominant effects of HS sequences controlled in the normal human globin gene locus? One possibility is that a choice must be made to determine which globin gene (ε, γ, or β) will interact with the upstream HS sequences (Fig. 1A). Competition between individual globin gene family members for interactions with the HS sequences could define temporal-specific expression. In this case, correct develop-

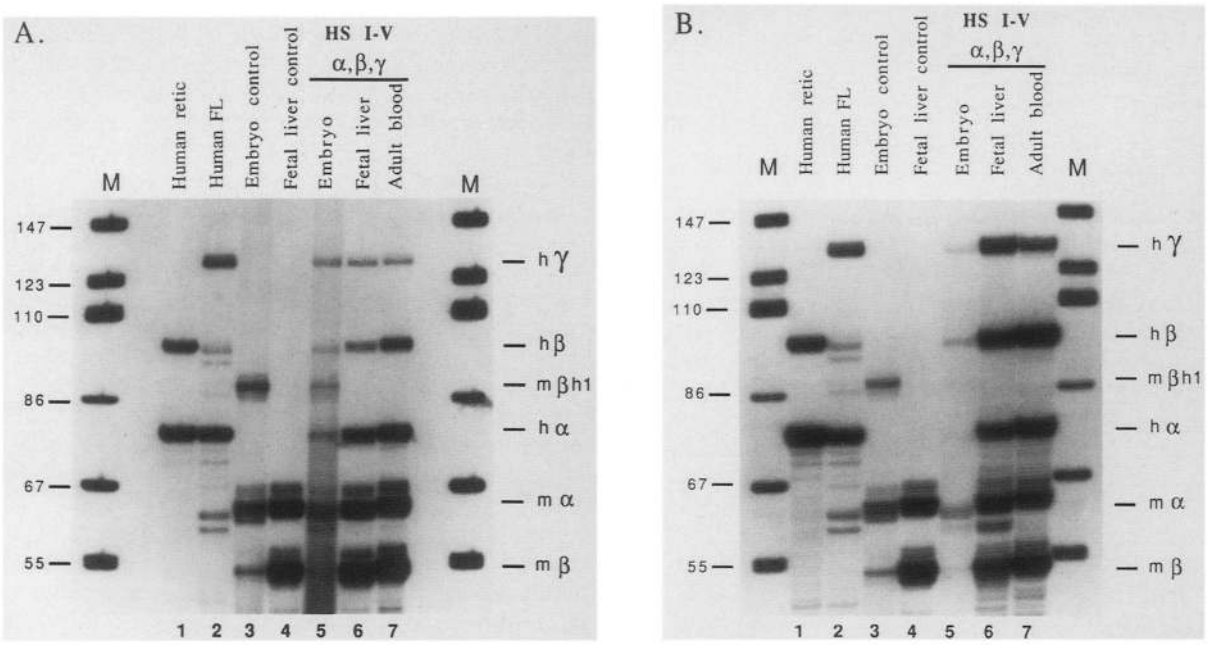

Figure 4. Primer extension analysis of RNA from 11-day embryos, 16-day fetal liver, and adult blood from two HS I–V α,β,γ (HS I–V α, HS I–V β, and HS I–V γ) transgenic mouse lines. Human γ-, β-, and α and mouse βh1-, α-, and β-globin-specific oligonucleotide primers were end-labeled and gel-purified. All six primers were hybridized with 20 μg of 11-day mouse embryo RNA, 5 μg of mouse fetal liver RNA, 1.0 μg of mouse blood cell RNA, 0.5 μg of human reticulocyte RNA, or 1.0 μg of human fetal liver RNA. Hybridized primers were then extended with reverse transcriptase to map the 5′ ends of human γ-, β-, and α- and mouse βh1-, α- and β-globin mRNA. (*A* and *B*) Two independently derived HS I–V α,β,γ transgenic mouse lines. (Lanes *1–4*) Human reticulocyte RNA, human fetal liver RNA, 11-day mouse embryo RNA, and 16-day mouse fetal liver RNA. (Lanes *5–7*) 11-day embryo RNA, 16-day fetal liver RNA, and adult blood RNA from HS I–V α,β,γ transgenic mice. Southern blots of tail DNA demonstrate that the transgenes are intact and linked as follows: ··· HS I–V α, HS I–V γ, HS I–V β, HS I–V γ ···. The order of the transgenes was random.

mental control should be restored in transgenic mice that contain human globin genes with different temporal specificities linked together with the HS sequences. To test this hypothesis, we coinjected a 22-kb DNA fragment containing HS I–V and a 40-kb fragment containing human ^{G}γ-, ^{A}γ-, ψβ-, δ-, and β-globin genes (designated γγψβδβ; Fig. 1B) into fertilized mouse eggs and implanted the eggs into the oviducts of foster mothers. Fetuses were removed at 16 days of development, and liver DNA was analyzed for intact copies of the transgenes by Southern blot hybridization with human γ-, β-globin, and HS-II-specific probes. Fetal liver RNA from all four animals that contained intact copies of the transgenes was then analyzed for human γ-, β-, and α-globin mRNA and mouse β-globin mRNA by primer extension analysis (Fig. 5). Lanes 1–3 are controls: human reticulocyte RNA, human fetal liver RNA, and mouse fetal liver RNA, respectively. Lanes 4–6 are fetal liver RNA from the three transgenic animals containing the human γ-globin gene alone. Lanes 7–9 are fetal liver RNA from three of the mice carrying HS I–V γ transgenes, and lanes 10–13 are fetal liver RNA from the four animals with the HS I–V γγψβδβ transgenes. All four of these animals expressed high levels of human β-globin mRNA but little human γ-globin mRNA. Two animals that contained the γγψβδβ transgene without the HS I–V fragment were also obtained; however, no expression of either the human γ- or β-globin gene was observed in these animals (see Fig. 6, lane 4).

The quantitative differences in human γ-globin gene expression in HS I–V γ and HS I–V γγψβδβ transgenic mice are listed in Table 1. The levels of human γ-globin mRNA in HS I–V γ animals averaged 26% of endogenous mouse β-globin mRNA per gene copy. In contrast, the average level of human γ-globin mRNA was 1% per gene copy in HS I–V γγψβδβ animals. This 26-fold reduction of human γ-globin gene expression in HS I–V

Table 1. *Human γ-globin gene expression in adult erythroid tissue of HS I–V γ and HS I–V γγψβδβ transgenic mice*

Transgene	Percent expression per gene copy[a] range	mean
HS I–V γ	7–77	26
HS I–V γγψβδβ	0–5	1

Human γ- and mouse β-globin mRNA levels in 16-day fetal liver were determined by solution hybridization with human γ- and mouse β-globin oligonucleotides (Townes et al. 1985b). The values of percent expression per gene copy were calculated as described by Ryan et al. (1989b). The ratio of human β to human γ in HS I–V γγψβδβ animals is 47% per gene copy.

[a] $\dfrac{\text{Human γ mRNA/human γ gene}}{\text{Mouse β mRNA/mouse β gene}} \times 100.$

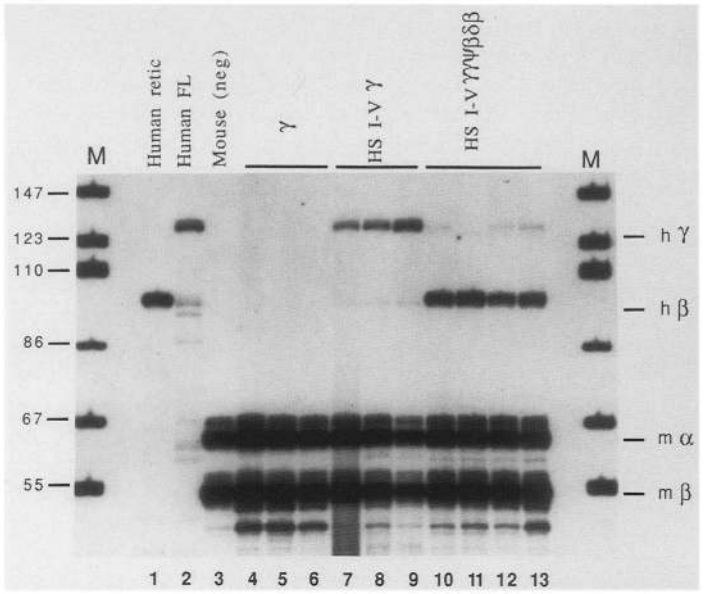

Figure 5. Primer extension analysis of 16-day fetal liver RNA from γ, HS I–V γ, and HS I–V γγψβδβ transgenic mice. Human γ- and β- and mouse α- and β-globin-specific oligonucleotide primers were end-labeled, gel-purified, hybridized to 5.0 μg of 16-day fetal liver RNA, and extended with reverse transcriptase. (Lane *1*) 0.5 μg of human reticulocyte RNA control; (lane *2*) 1.0 μg of human fetal liver RNA control; (lane *3*) fetal liver RNA from a mouse control; (lanes *4–6*) fetal liver RNA from transgenic mice containing the human ^{A}γ-globin gene alone (same as lanes *3–5* of Fig. 3); (lanes *7–9*) fetal liver RNA from HS I–V γ transgenic mice (same as lanes *7–9* of Fig. 3); (lanes *10–13*) fetal liver RNA from HS I–V γγψβδβ transgenic mice (see Fig. 1B). Southern blots of fetal liver DNA of all four HS I–V γγψβδβ animals demonstrate that the HS I–V and γγψβδβ transgenes are intact (see Methods) and suggest the following general structure: ··· HS I–V, HS I–V, HS I–V ··· γγψβδβ, γγψβδβ, γγψβδβ ··· or ··· HS I–V, HS I–V, HS I–V ··· βδψβγγ, βδψβγγ, βδψβγγ ···. The number of HS I–V transgenes per cell in the samples represented in lanes *10–13* is 3, 3, 2, and 6, respectively; the number of γγψβδβ transgenes per cell is 2, 10, 2, and 8, respectively. The exact structure of the junctions between HS I–V and γγψβδβ transgenes is not known.

γγψβδβ mice compared to that in HS I–V γ animals is consistent with the competitive effects of a linked β-globin gene on γ-globin expression. The difference in human γ- and β-globin gene expression in HS I–V γγψβδβ mice also illustrates this effect. The average level of human β-globin mRNA was 47% of endogenous mouse β-globin mRNA per gene copy. This 47-fold difference in human γ- and β-globin gene expression in adult erythroid tissue of HS I–V γγψβδβ mice demonstrates that correct temporal specificity can be attained when both human γ- and β-globin genes are linked with the HS sequences.

To determine whether human γ- and β-globin genes are also regulated correctly in embryonic development, the same HS I–V and γγψβδβ fragments were coinjected into fertilized eggs, and expression was analyzed in 11-day embyros (Fig. 6). Lanes 1 and 2 are human fetal liver RNA and human reticulocyte RNA controls, respectively. Lanes 3 and 4 are 11-day embryo RNA and 16-day fetal liver RNA, respectively, from animals that contain only the γγψβδβ fragment; no human γ- or β-globin mRNA is observed in these samples or in nine other 11-day embryos containing γγψβδβ alone. Lanes 5–7 are three independently derived embryos containing both the HS I–V and γγψβδβ fragments. All three animals expressed as much or more human γ-globin mRNA as mouse embryonic (βh1) mRNA; however, no human β-globin mRNA could be detected in these animals even though the β-globin transgenes were intact (data not shown). For comparison, lanes 8–11 are 16-day fetal

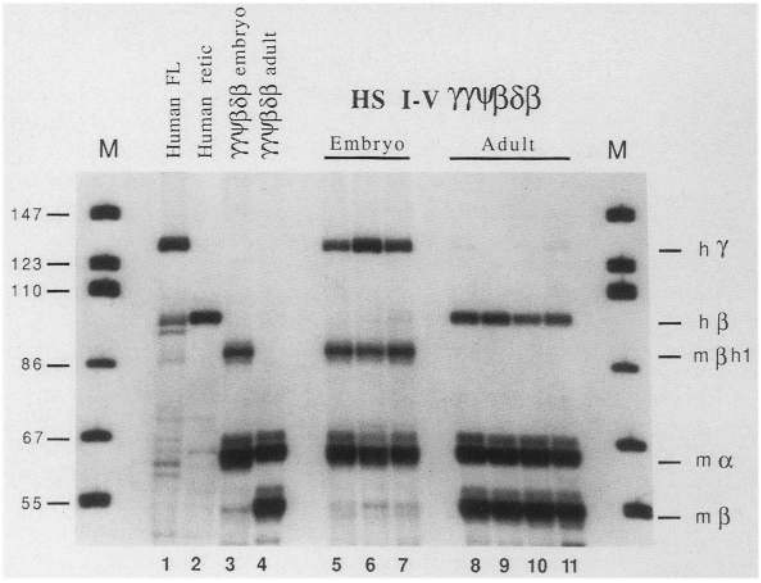

Figure 6. Primer extension analysis of RNA from embryonic and adult erythroid tissue of HS I–V γγψβδβ transgenic mice. Human γ- and β- and mouse βh1-, α-, and β-globin-specific oligonucleotide primers were end-labeled, gel-purified, hybridized to 10 μg of 11-day embryo RNA or 5.0 μg of 16-day fetal liver RNA, and extended with reverse transcriptase. (Lane *1*) 1.0 μg of human fetal liver RNA control; (lane *2*) 0.5 μg of human reticulocyte RNA control; (lanes *3* and *4*) 11-day embryo RNA and 16-day fetal liver RNA, respectively, from mice that contain only the γγψβδβ fragment; (lanes *5–7*) RNA from three independently derived 11-day embryos containing both the HS I–V and γγψβδβ fragments; (lanes *8–11*) 16-day fetal liver RNA from the same HS I–V γγψβδβ animals that were analyzed in Fig. 5 (lanes *10–13*). Southern blots of embryo DNA and fetal liver DNA demonstrate that the transgenes are intact and arranged as described in the legend to Fig. 5. The number of γγψβδβ transgenes per cell in lanes *3–7* are 4, 10, 5, and 15, respectively. The number of HS I–V transgenes per cell in the samples represented in lanes *5–7* are 2, 1, and 2, respectively.

liver RNAs from the same HS I–V γγψβδβ animals that were analyzed in Figure 5 (lanes 10–13). As described above, human γ-globin genes are virtually silent and β-globin genes are expressed at high levels, as expected for correct switching.

γ-Globin expression in HS I–V $^{G}\gamma^{A}\gamma\psi\beta$ animals

Inhibition of human γ-globin expression in adult erythroid tissue of HS I–V γγψβδβ mice could result from competitive effects of the human β-globin gene or from repression by sequences located between the fetal (γ) and adult (δβ) globin genes. To distinguish between these possibilities, a 27.5-kb fragment containing $^{G}\gamma^{A}\gamma\psi\beta$ (designated γγψβ; Fig. 1B) was coinjected with HS I–V into fertilized eggs and γ-globin mRNA was analyzed in 16-day fetal liver. Two animals that contained intact copies of both trangenes were obtained, and the primer extension results are illustrated in Figure 7. Lane 1 is a human fetal liver RNA control. Lanes 2 and 3 are 16-day fetal liver RNA from two animals, which contained the γγψβ fragment alone. No γ expression was observed in these two animals that contained the γγψβ fragment without the HS I–V fragments. Lanes 4 and 5 are the two mice that contained both HS I–V and γγψβ transgenes. Both animals expressed correctly initiated human γ-globin mRNA in this adult erythroid tissue, and the average level of expression was 24% of endogenous mouse β-globin mRNA per transgene copy. These results demonstrate that the intergenic region between γ and δβ does not contain sequences sufficient to silence human γ-globin gene expression in adult erythroid tissue.

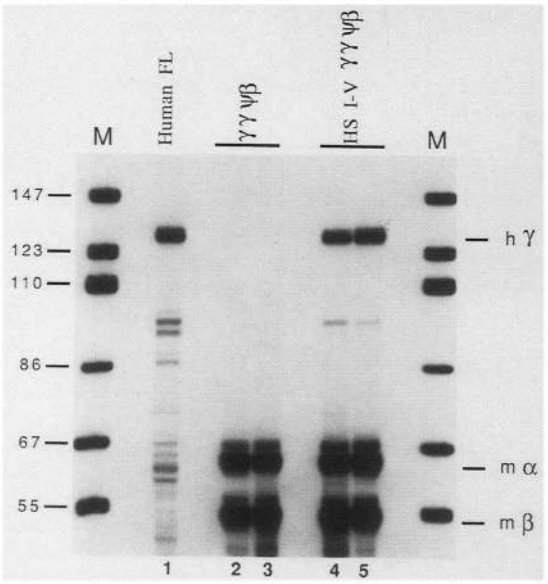

Figure 7. Primer extension analysis of 16-day fetal liver RNA from HS I–V γγψβ transgenic mice. Human γ- and mouse α- and β-globin-specific oligonucleotide primers were end-labeled, gel-purified, hybridized to 16-day fetal liver RNA, and extended with reverse transcriptase. (Lane *1*) 1.0 μg of human fetal liver RNA; (lanes *2* and *3*) 5.0 μg of 16-day fetal liver RNA from mice containing the γγψβ transgene alone; (lanes *4* and *5*) 5.0 μg of 16-day fetal liver RNA from two mice that contain both HS I–V and γγψβ transgenes. Southern blots of fetal liver DNA demonstrate that the transgenes are linked as described in the legend to Fig. 5. The number of γγψβ transgenes per cell in lanes *2–5* are 1, 3, 10, and 10, respectively. The number of HS I–V transgenes per cell in the samples represented in lanes *4* and *5* is 2 and 3, respectively.

Discussion

Figure 8 summarizes the results described above. Human γ- and β-globin genes without HS sequences were expressed with correct temporal specificity in transgenic mice, but the levels of expression were low (Fig. 8A,B). When the genes were inserted individually downstream of the HS sequences, expression was increased to high levels but developmental specificity was lost (Fig. 8C,D). Correctly regulated γ-globin expression was not observed even when 13.8-kb of $^{A}\gamma$3′-flanking sequence was included in the injected fragment (Fig. 8E). In contrast, correct temporal regulation of human γ- and β-globin genes was restored when a 40-kb fragment containing $^{G}\gamma$-$^{A}\gamma$-ψβ-δ-β was tested with the HS sequences (Fig. 8F).

Model for globin gene regulation

Figure 9 illustrates a general model for globin gene switching that is consistent with the results summarized in Figure 8. The DNase I HS sequences are activated in early erythroid cell precursors and presumably organize the entire β-globin locus into an open chromatin domain that is stable throughout development. Once the open domain is established, the HS sequences function as a powerful enhancer to direct high levels of globin gene expression. In this model, correct temporal regulation results from competition of individual globin gene family members for stable interactions with the HS sequences. Promoter and proximal enhancer-binding factors synthesized in yolk sac, fetal liver, and bone marrow could influence these competitive interactions either positively or negatively and subsequently determine developmental specificity.

Correct temporal regulation is observed when human γ- and β-globin genes without HS sites are analyzed in transgenic mice (Costantini et al. 1985; Townes et al. 1985b; Chada et al. 1986; Kollias et al. 1986). The human γ gene with 1300 bp of 5′-flanking and 370 bp of 3′-flanking sequence is expressed with the same developmental specificity as the mouse embryonic genes; expression is activated in yolk-sac-derived erythroid cells during embryonic development, and the gene is turned off when the major site of erythropoiesis shifts to the fetal liver. The human β-globin gene is expressed with the opposite developmental specificity. Human β-globin transgenes with as little as 815 bp of 5′-flanking and 1700 bp of 3′-flanking sequence are not expressed in yolk-sac-derived erythroid cells but are activated concomitantly with the endogenous mouse β-globin genes in fetal liver and bone marrow. These data suggest that

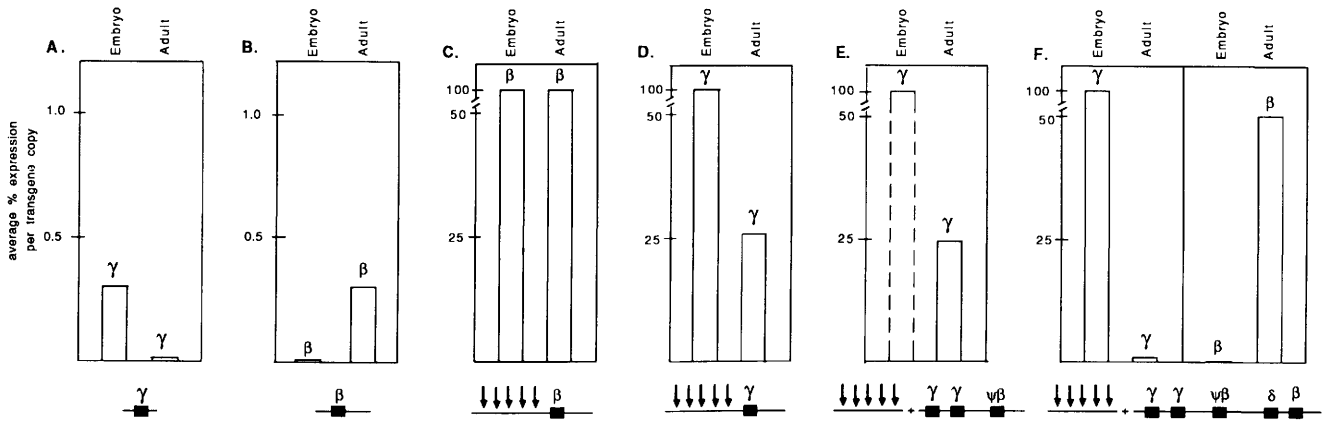

Figure 8. Summary of data in Figs. 2–7. The average percent expression per transgene copy of human γ- and β-globin genes compared to that of endogenous embryonic or adult mouse β-globin genes is illustrated. Human γ- and β-globin genes are expressed with correct tissue and temporal specificity in transgenic mice, but the levels of expression are low (*A* and *B*). When HS sequences are inserted immediately upstream of the genes, expression is increased to normal levels, but temporal specificity is lost (*C–E*). However, developmental specificity is restored when γ- and β-globin genes on the same fragment are tested with the HS sequences (*F*). The broken line bar in *E* represents an inferred level of human γ-globin gene expression in HS I–V γγψβ transgenic embryos.

sequences within or immediately flanking the genes determine temporal specificity. Sequences located within and downstream of the human β-globin gene activate reporter gene expression in erythroid tissues of transgenic mice at the correct developmental stage (Behringer et al. 1987; Kollias et al. 1987; Trudel et al. 1987; Magram et al. 1989). Therefore, these sequences appear to be involved in temporal regulation. Interestingly, human γ/β-globin hybrid genes are expressed in both embryonic and adult erythroid tissue in transgenic mice (Kollias et al. 1986; Behringer et al. 1987; Trudel et al. 1987). Presumably, the 5′ end of the γ-globin gene contains sequences that determine embryonic expression, and β-globin 3′ sequences determine adult expression.

If competition between ε-, γ-, and β-globin genes for HS sequences is an important aspect of temporal control, individual genes inserted downstream of the HS sites should not be correctly regulated during development. The results described in Figures 2–4 demonstrate that correct temporal regulation is lost when human γ- or β-globin genes are individually fused downstream of the HS sequences. High levels of human γ- and β-globin expression are observed in embryonic, fetal, and adult erythroid tissues. Although yolk sac, fetal liver, and bone marrow regulatory factors are presumably present in appropriate concentrations, correct temporal control is not achieved.

One critical test of the model is to link the HS sequences with two globin genes that are differentially regulated during development and to determine whether

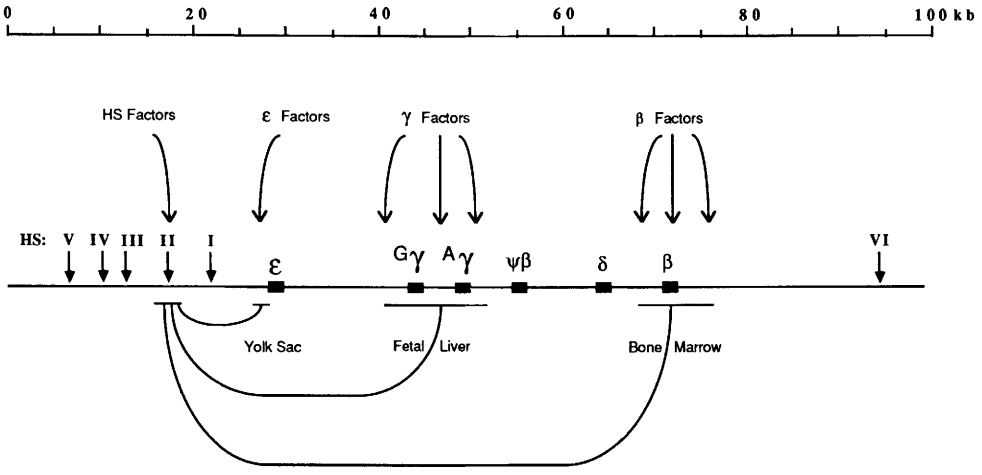

Figure 9. General model for human hemoglobin switching. In this model correct temporal regulation results from competition of individual globin gene family members for interaction with HS sequences. Promoter and proximal enhancer binding factors synthesized in yolk sac, fetal liver, and bone marrow influence these competitive interactions either positively or negatively and subsequently determine developmental specificity. For clarity, only HS II is shown to interact with ε-, γ-, or β-globin genes, but HS III, IV, and/or V may also be involved (Ryan et al. 1989b).

the genes are expressed with the correct temporal specificity. Figures 5 and 6 demonstrate that the human γ-globin gene is expressed at high levels only in embryonic tissue and the human β-globin gene is expressed at high levels only in adult erythroid tissue in HS I–V γγψβδβ mice. Therefore, correctly regulated expression is restored when human γ- and β-globin genes are linked with the HS sequences. These results suggest that the HS sequences, themselves, do not control globin gene switching during human development but that they interact with sequences surrounding individual genes to direct high-level, temporal-specific expression. Regulatory proteins that are synthesized or activated at defined developmental stages could bind to promoter and/or proximal enhancer sequences and provide the ε-, γ-, or β-globin genes with a selective advantage for stable interactions with HS-bound factors. Alternatively, repressors that inhibit interactions between specific globin gene family members and the HS sequences could preferentially silence genes at different developmental stages. In this model, the 35 kb of DNA between γ- and β-globin genes is likely to play a minor role in switching. If there is an influence of these sequences, it may be to provide additional refinement of competitive interactions or to play an important spacing function, thereby preventing interference of one active gene with another. These are all testable predictions.

Conclusion

The data described in this paper strongly support a model of human globin gene switching that is based on competition of globin gene family members for interactions with the DNase I HS sequences located upstream of the locus. The HS sequences presumably form highly stable complexes with individual globin genes and therefore commit cells to synthesize embryonic, fetal, or adult hemoglobins. Regulatory factors present in yolk sac, fetal liver, and bone marrow may bind sequences within and/or surrounding ε-, γ-, and β-globin genes and determine which genes will interact with the powerful HS enhancer. Once a stable interaction is initiated, expression is maintained at a high level throughout the transcriptional lifetime of the cell.

Methods

Construction of HS α-, HS β-, and HS γ-globin clones

The construction of HS I–V β, HS I,II β, and HS I,II α clones was described previously (Ryan et al. 1989a,b). HS I–V α and HS I–V γ cosmids were constructed exactly as described for HS I–V (22) β (Ryan et al. 1989b), except that a 3.8-kb *Bgl*II–*Eco*RI fragment containing the human α1-globin gene or a 3.2-kb *Hin*dIII fragment containing the human ^{A}γ-globin gene was inserted into the right arm vector. The HS I–V fragment used for coinjection experiments was a 22-kb *Sal*I–*Cla*I fragment isolated from HS I–V (22) β. The γγψβδβ fragment was a 40-kb *Kpn*I fragment isolated from a cosmid kindly provided by R. Gelinas, and γγψβ was a 27.5-kb *Kpn*I–*Sal*I fragment derived from the same cosmid clone. The β- and ^{A}γ-globin genes without HS sites were contained on 4.1-kb *Hpa*I–*Xba*I and 3.2-kb *Hin*dIII fragments, respectively.

Sample preparation and microinjection

All of the constructs were removed from vector sequences by digestion with the appropriate enzymes and isolated on low-gelling-temperature agarose (FMC) gels. The fragments were purified as described (Ryan et al. 1989b) and microinjected into the pronuclei of F_2 hybrid eggs from C57BL6/SJL parents, as described by Brinster et al. (1985). When multiple constructs were injected, the fragments were mixed in a 1 : 1 molar ratio. Injected eggs were implanted into the uteri of foster mothers, and animals were removed at 11 or 16 days, or allowed to develop to adults.

DNA analysis

Total nucleic acids were isolated from whole 11-day embryos, 16-day fetal livers, or tails by SDS–proteinase K treatment, followed by phenol/chloroform extraction (Brinster et al. 1985). Samples that contained the transgenes were determined by DNA dot hybridization with the HS, α-, β-, and γ-globin probes described previously (Townes et al. 1985b; Behringer et al. 1987; Ryan et al. 1989a,b). Southern blots to determine transgene structure and copy number were performed as described (Ryan et al. 1989b). Fetal liver DNA samples from HS I–V γγψβδβ and HS I–V γγψβ animals were cut with *Bgl*II, and blots were probed with a 1.9-kb *Hin*dIII fragment containing HS II. The 13.0- and 7.7-kb *Bgl*II fragments predicted for head-to-tail tandem arrays of HS I–V were observed in all samples. Fetal liver samples from γγψβδβ and HS I–V γγψβδβ animals were cut with *Bam*HI, and blots were probed with a 790-bp *Hin*fI fragment from β intervening sequence (IVS) 2. The 9.7-kb fragment predicted for head-to-tail tandem arrays of γγψβδβ was detected in all samples. Fetal liver samples from γγψβ and HS I–V γγψβ animals were cut with *Bam*HI, and blots were probed with a 456-bp *Bam*HI–*Pvu*II fragment from γ IVS 2. The 20- and 4.9-kb fragments predicted for head-to-tail tandem arrays of γγψβ were observed in all samples.

RNA analysis

Eleven-day embryo RNA and 16-day fetal liver RNA were prepared from total nucleic acids as described (Ryan et al. 1989b). Blood RNA was prepared from frozen cell pellets by the method of Chomczynski and Sacchi (1987), with the following modification. The final RNA pellets were resuspended in a solution containing 1.0% SDS, 100 μg/ml proteinase K, 25 mM NaCl, 1.0 mM EDTA, and 10 mM Tris-HCl (pH 7.5). After digestion for 3 hr at 50°C, the samples were extracted with phenol/chloroform and chloroform and were ethanol-precipitated. Human α, β, and γ-globin, and mouse β-globin mRNA levels were quantitated by solution hybridization with oligonucleotide probes, as described previously (Townes et al. 1985b; Behringer et al. 1987; Ryan et al. 1989a,b). Primer extensions were performed as described by Townes et al. (1985), except that the samples were boiled for 5 min immediately before hybridization. The human γ primer 5′-CCAGCATCTTCCACATTCACC-3′ corresponds to sequences from +107 to +127 of the human γ-globin gene. The human β primer 5′-AGACGGCAATGACGGGACACC-3′ corresponds to sequence from +78 to +98 of the human β-globin gene. The human α primer 5′-GGCCTTGACGTTGGTCTTGTCGGCAGG-3′ corresponds to sequence from +50 to +76 of the human α-globin gene. The mouse α primer 5′-CAGGCAGCCTTGATGTTGCTT-3′ corresponds to sequences from +45 to +65 of the mouse α-globin gene. The mouse β primer 5′-TGATGTCTGTTTCTGGGGTTGTG-3′ corresponds to sequences from +31 to +53 of the mouse β-globin gene. The mouse βh1 primer 5′-ATAGCTGCCTTC-

TCCTCAGCT-3′ corresponds to sequences from +67 to +87 of the mouse βh1-globin gene.

Acknowledgments

We gratefully acknowledge the excellent technical assistance of Janine Askins, Nan Martin, and Mary Avarbock, and we especially thank Kevin Pawlik for the primer extension gels illustrated in Figure 3 and Figure 4B. We also thank Eric Sandgren, David Lo, and Gary Hoyle for help with experiments. We thank Rich Gelinas for the γγψββδβ cosmid clone and Jeff Engler for synthesis of oligonucleotides. This work was supported, in part, by grants HL-35559, HD-09172, and HD-23657 from the National Institutes of Health. T.M.R. is a predoctoral trainee supported by National Institutes of Health grant T32 CA-09467

Note added in proof

Enver et al. [*PNAS* **86:** 7033–7037, 1989] recently described HS γ transgenic mice that expressed the human ^{A}γ-globin gene in both embryonic and adult erythroid tissue.

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