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Developmental Specificity of the Interaction between the Locus Control Region and Embryonic or Fetal Globin Genes in Transgenic Mice with an HS3 Core Deletion

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The human β-globin locus control region (LCR) consists of five erythroid-lineage-specific DNase I-hypersensitive sites (HSs) and is required for activation of the β-globin locus chromatin domain and globin gene expression. Each DNase I-HS of the LCR consists of a highly conserved core element and flanking sequences. To analyze the functional role of the core elements of the HSs, we deleted a 234-bp fragment encompassing the core of HS3 (HS3c) from a β-globin locus residing on a 248-kb β-locus yeast artificial chromosome and analyzed its function in F_2 progeny of transgenic mice. Human ε-globin gene expression was absent at day 10 and severely reduced in the day 12 embryonic erythropoiesis of mice lacking HS3c. In contrast, γ-globin gene expression was normal in embryonic erythropoiesis but it was absent in definitive erythropoiesis in the fetal liver. These results indicate that the core element of HS3 is necessary for ε-globin gene transcription in embryonic cells and for γ-globin gene transcription in definitive cells. Normal γ-globin gene expression in embryonic cells and the absence of γ-globin gene expression in definitive cells show that different HSs interact with γ-globin gene promoters in these two stages of development. Such results provide direct evidence for developmental stage specificity of the interactions between the core elements of HSs and the promoters of the globin genes.

The human β-globin locus contains five actively transcribed genes that are arranged in their developmental order of expression. High-level expression of the β-globin gene cluster is dependent on the presence of the locus control region (LCR) (18), an element characterized by a series of five DNase I-hypersensitive sites (HSs) located 6 to 22 kb upstream of the ε-globin gene (9, 10, 18, 44). Naturally occurring deletions of this element result in changes in chromatin structure that extend at least 200 kb 3′ of the deletion, transcriptional silencing of the β-globin locus, and a phenotype of β thalassemia (4, 5, 12, 22). Functional properties of the LCR include activation of the β-globin locus (10, 18), restriction of globin gene expression to cells of the erythroid cell lineage (18, 45), enhancement of globin gene expression (11, 18, 39), and protection from position effects of globin genes transferred in transgenic mice (13, 18, 25, 41).

Transgenic mice have been extensively used to study the developmental control of the β-globin genes, the function of the LCR, and the role of individual HSs in β-globin gene regulation. Linkage of individual HSs to individual globin genes have shown that HS2, HS3, and HS4 are capable of conferring position-independent expression of globin genes, with stronger activation of expression at a specific stage of development (14, 25). Several observations have led to a model suggesting that the HSs form a complex that directly interacts with globin gene promoters by looping of the intervening DNA (7, 28, 46). HS2, HS3, and HS4 have 200- to 400-bp core regions that are able to provide position-independent expression in transgenic mice (27, 34, 35, 37, 42). These HS core regions may be indispensable components of the LCR complex; deletions of the HS3 or HS4 **core** elements result in disruption of HS function and reduction of globin gene expression (3).

Discernment of the function of individual HSs and analysis of how the LCR interacts with individual genes during development require studies in the context of intact, native β-globin loci. Entire β-globin loci have been used to generate transgenic mice, by ligating two cosmids to produce a 70-kb fragment (40) or by using 248-kb (30) or 150-kb (15, 36) yeast artificial chromosomes harboring the β-globin locus (β-YACs). Mice carrying β-YACs show correct regulation of the human globin genes, presumably because all the human *cis*-regulatory elements are present in the transferred sequences of the β-globin locus and are properly recognized by the murine *trans*-acting environment. In β-YAC transgenic mice, the ε-globin gene is expressed during the embryonic stage of development and is confined to primitive erythropoiesis in the yolk sac. The γ-globin genes are also expressed in the embryonic yolk sac, but unlike their murine homologous gene, βh1, γ-globin gene expression continues in the fetal liver stage of erythropoiesis. Human β-globin gene expression occurs only in the cells of definitive erythropoiesis.

To delineate the role of HS3 in LCR function and globin gene expression during development, we produced β-YAC transgenic mice carrying either large deletions of LCR sequences containing the individual HSs or the core elements of these sites. We have previously reported results obtained from extensive deletions of HS3 and HS2 (32). In the study summarized in this paper, we deleted the core element of HS3 of the LCR from a β-globin locus residing on a 248-kb β-YAC and used this β-YAC to produce transgenic mice. In the embryonic yolk sac of these mice, ε-globin gene expression was absent but γ-globin gene expression was normal. However, γ-globin gene

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expression was totally silent in erythroid cells originating in fetal liver. The levels of β-globin gene expression were decreased and varied among the transgenic lines, indicating that β-globin gene expression was influenced by the position of integration of the β-YAC transgene into the murine genome. Based on these results, we propose that the core of the HS3 directly interacts with the globin gene promoters during embryonic and fetal development, resulting in activation of ε-globin gene expression in the yolk sac and of γ-globin gene expression in the fetal liver. Indirectly, our results also suggest that the LCR changes conformations during the course of development.

MATERIALS AND METHODS

Construction of a β-YAC lacking HS3c (ΔHS3c). Plasmid pIII(0.7) contains a 784-bp *Pst*I fragment encompassing the 225-bp 5′ HS3c element and 559 bp of flanking DNA sequence (GenBank coordinates 4348 to 5132). Plasmid pIII(0.7) DNA was digested with the restriction enzyme *Pst*I, and the 784-bp insert was subcloned into *Pst*I-digested plasmid pALTER-1 (Promega, Madison, Wis.) to generate pALHS3(0.7). Two primers, each with two nucleotide substitutions, were synthesized and used to create *Eco*RI sites flanking the 5′ HS3c element (GenBank coordinates 4541 to 4776) by site-directed mutagenesis with the Altered Sites II in vitro mutagenesis system (Promega) according to the manufacturer's protocol. The primer DNA sequences were 5′ CCCTCACGGTGAATT**C**GCGAGCTGG 3′ (proximal) and 5′ GTAGTAGAATGAA**G**AAT**C**TGCTA TGC 3′ (distal); the nucleotide substitutions are in boldface type and the *Eco*RI sites are underlined. Plasmid pIII(4.4), containing a 225-bp *Hpa*I-*Kpn*I fragment encompassing 5′ HS3 (GenBank coordinates 3379 to 7764), was digested with restriction enzyme *Hin*dIII, and a 1.8-kb *Hin*dIII fragment containing 5′ HS3 sequence was isolated and subcloned into *Hin*dIII-digested pUC19 in which the *Eco*RI and *Pst*I sites had been ablated. The resultant plasmid, pUCHS3(1.8), which contained 5′ HS3 (GenBank coordinates 3379 to 5172), was digested with restriction enzyme *Pst*I to remove the 784-bp *Pst*I fragment containing 5′ HS3 sequence, and the mutagenized 784-bp fragment from plasmid pALHS3(0.7) was subcloned in its place to generate plasmid pUCHS3m. Plasmid pUCHS3m was digested with restriction enzyme *Eco*RI to remove the 234-bp *Eco*RI fragment containing the 5′ HS3c element and circularized by the addition of T4 DNA ligase (Boehringer Mannheim, Indianapolis, Ind.) to generate plasmid pUCΔHS3c (1.6). Digestion of pUCΔHS3c(1.6) with restriction enzyme *Hin*dIII generated a 1.6-kb *Hin*dIII fragment containing the 5′ HS3 core deletion that was subcloned into the yeast-integrating-plasmid (YIP) vector pRS406 (Stratagene, La Jolla, Calif.), from which the *Spe*I restriction site had been deleted to produce plasmid pRSΔHS3c(1.6). One microgram of pRSΔHS3c(1.6) was linearized with *Spe*I at a unique site 3′ of the 5′ HS3 deletion and transformed into yeast spheroplasts (16). Transformants were selected for uracil prototrophy on complete medium lacking uracil, and proper intergration of the YIP in isolates containing YACs was determined by Southern blot hybridization analysis. Spontaneous excision of the YIP via homologous recombination was permitted by overnight growth in nonselective rich medium (yeast-peptone-dextrose) (47). Aliquots of the culture were plated on 5-fluoroorotic acid plates to select for loss of the URA3 gene due to excision of the YIP vector, which resulted in 5-fluoroorotic acid resistance. Deletion of the 5′ HS3c element was determined by Southern blot hybridization analysis. (The approach used is summarized in Fig. 1.)

YAC purification and production of transgenic mice. The ΔHS3c β-YAC yeast strain was grown and agarose plugs were prepared as previously described (20). Preparative plugs were loaded on a 0.5% MP agarose gel (Boehringer Mannheim), and the DNA was fractionated by pulsed-field gel electrophoresis (PFGE) (CHEF DRII apparatus; Bio-Rad, Hercules, Calif.) in 0.5× TBE (44.5 mM Tris, 44.5 mM boric acid, 1 M EDTA) at 200 V with a 60-s switch for 20 h at 12°C. A portion of the gel was stained with ethidium bromide to determine the migration distance of the ΔHS3c β-YAC, and the YAC DNA was cut from the gel. The gel slice containing the YAC DNA and a second slice containing a yeast chromosome were rotated 90° relative to their original directions of mobility and electrophoresed in a 4% low-melting-point agarose (LMPA) (NuSieve GTG; FMC, Rockland, Maine) in 0.5× TBE at 47 V for 15 h to concentrate the YAC. The yeast lane was stained with ethidium bromide to determine the migration distance of the β-YAC DNA into the 4% LMPA. A slice of approximately 8 mm was weighed and equilibrated in a 100× volume of 10 mM Tris-HCl (pH 7.5)–250 μM EDTA–100 mM NaCl for 1 h at room temperature without agitation. The gel slice was placed in a microcentrifuge tube, and the agarose was melted at 68°C for 10 min and then immediately placed at 42.5°C for 5 min. Two units of β-agarase (New England Biolabs, Beverly, Mass.) per 100 mg of agarose was added and digested overnight at 42.5°C. Integrity of the YAC DNA was determined by PFGE as described above prior to its injection into fertilized mouse eggs. DNA concentration was determined by fluorometry (Pharmacia, Piscataway, N.J.), and the YAC DNA was diluted to a final concentration of 2.0 ng/μl with a solution containing 10 mM Tris-HCl (pH 7.5), 250 μM EDTA, and 100 mM NaCl and filtered through a 0.22-μm-pore-size Acrodisk (Gelman, Ann Arbor, Mich.) just prior to injection.

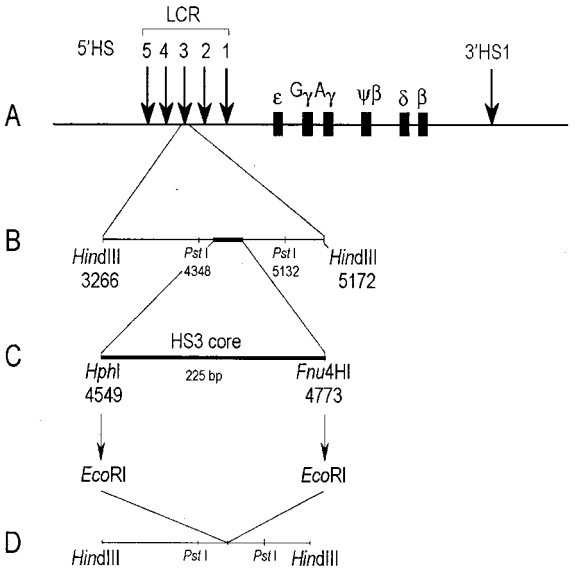

FIG. 1. β-Globin locus and the location of the HS3c deletion. (A) Five DNase I-HSs are located 6 to 22 kb 5′ of the ε-globin gene, and a single HS (3′ HS1) is located approximately 20 kb 3′ of the β-globin gene. (B) A 784-bp *Pst*I fragment containing the HS3c was subcloned into pALTER-1 for site-directed mutagenesis. (C) The *Hph*I and *Fnu*4HI restriction sites, flanking the core element of HS3, were mutagenized to create *Eco*RI sites. Digestion with *Eco*RI simultaneously eliminated the core element of HS3 and left a diagnostic *Eco*RI restriction site. (D) The 1.6-kb *Hin*dIII fragment was subcloned into the YIP used to introduce the deletion into the β-YAC via homologous recombination.

Structural analysis of ΔHS3c β-YAC transgenic mice. Transgenic founder (F_0) animals were identified by hybridization of tail DNA slot blots with a γ-globin gene probe. Founders were bred to produce F_1 progeny for structure-function analysis. Fresh liver cell suspensions were prepared as follows. Liver was cut into small pieces and then mechanically sheared by successive passage through a 16-gauge syringe. The cells were washed twice with Dulbecco's phosphate-buffered saline and resuspended at a concentration of 3×10^7 cells/ml in phosphate-buffered saline. An equal volume of 2% LMPA (Seaplaque GTG agarose) was added to the liver suspension, and plugs were cast. The plugs were incubated in LDS solution (1% lithium dodecyl sulfate, 100 mM EDTA [pH 8.0], 10 mM Tris-HCl [pH 8.0]) at 37°C for 1 h, followed by a second incubation overnight. The plugs were then washed twice for 30 min in 0.2× NDS (0.2% lauryl sarcosinate, 100 mM EDTA, 2 mM Tris base [pH 9.5]), followed by three 30-min washes in TE (10 mM Tris-HCl [pH 8.0], 1 mM EDTA [pH 8.0]). The plugs were stored at 4°C in TE.

Twelve agarose plugs were digested overnight at 50°C with 20 U of *Sfi*I (Boehringer Mannheim) in a total volume of 200 μl after preequilibration in 200 μl of 1× *Sfi*I buffer. The DNAs were fractionated by PFGE with a 1% agarose gel (SeaKem Gold GTG; FMC) at 200 V with a 14-s switch for 22 h at 14°C in 0.5× TBE. The gels were capillary blotted overnight onto nylon membranes (Hybond N^+; Amersham, Arlington Heights, Ill.) in 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). The nylon membrane was cut into strips representing individual lanes, and each strip was hybridized with a different probe spanning the β-globin locus from 5′ HS3 to the hereditary persistence of fetal hemoglobin type 6 (HPFH6) breakpoint. After overnight hybridization and washing, the strips were reassembled and subjected to autoradiography. A 140-kb *Sfi*I fragment is the expected size for an intact ΔHS3c β-YAC, and fragments of different sizes indicate that ΔHS3c β-YAC copies have deletions. The probes used were as follows: 0.7-kb *Pst*I 5′ HS3, 1.9-kb *Hin*dIII 5′ HS2, the 3.7-kb *Eco*RI ε-globin gene, the 2.4-kb *Eco*RI 3′ ^{A}γ-globin gene, 1.0-kb *Eco*RV ψβ, the 2.1-kb *Pst*I 5′ δ-globin gene, the 0.9-kb *Eco*RI-*Bam*HI β-globin gene, 1.4-kb *Xba*I DF10 (3′ HS1), 1.9-kb *Bgl*II HPFH3, 0.5-kb *Hin*dIII H500, and 1.5-kb *Eco*RI-*Bgl*II HPFH6. All fragments were radiolabeled with a Decaprime II random labeling kit (Ambion, Austin, Tex.). The 5′ δ-globin gene, HPFH3, and HPFH6 probe templates were gifts of N. P. Anagnou (University of Crete), DF10 was a gift from D. Fleenor (Duke University), and H500 was a gift from D. Mager (University of British Columbia).

Copy number determination. Agarose plugs containing transgenic mouse liver DNA were digested overnight with the restriction enzyme *Acc*I, and the DNAs were fractionated by agarose gel electrophoresis and blotted to a nylon membrane as described above. Copy number was determined by comparing human

$^{A}\gamma$-globin gene and murine Thy1.1 (gift from R. Perlmutter) hybridization signals by Southern blot hybridization. Thy1.1 serves as an internal diploid control. To ensure equal levels of labeling of both the $^{A}\gamma$-globin gene and Thy1.1 fragments, the following construct was synthesized. A 753-bp *Hin*dIII fragment containing sequences 3′ of the $^{A}\gamma$-globin gene (GenBank coordinates 41382 to 42135) was cloned into pW126, a pBluescript (Stratagene) plasmid containing a 544-bp *Bam*HI Thy1.1 cDNA, to produce pThy1.1/3′ $^{A}\gamma$(753). Digestion with *Xba*I and *Xho*I released a 1.3-kb fragment that was labeled with a Decaprime II random labeling kit (Ambion). As an internal control during Southern blot hybridization, we digested pThy1.1/3′ $^{A}\gamma$(753) with *Pst*I and *Sca*I to release a 2.6-kb $^{A}\gamma$ fragment and a 1.6-kb Thy1.1 fragment. Approximately 10 pg of this control was electrophoresed alongside the digested mouse genomic DNAs. Hybridization signals were quantitated with a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.). The ratio of $^{A}\gamma$ to Thy1.1 was calculated from the plasmid control, and this ratio was used to correct for differences in specific activities between the two probes. The corrected Thy1.1 values were divided by 2 to obtain a single copy value for each lane containing genomic DNA. ΔHS3c β-YAC copy number was determined by dividing the $^{A}\gamma$ signal by the corrected Thy1.1 single-copy value.

Measurement of globin mRNA synthesis. Total RNA was isolated from F_2 transgenic tissues with the RNAgents Isolation system (Promega). Human and murine globin RNA were quantitated by RNase protection analysis with an RPA II kit (Ambion). RNA probes were synthesized with a MAXIscript transcription kit (Ambion). Template DNAs used to measure human ε-, γ-, and β-globin mRNAs were pT7H ε(188), pT7$^{A}\gamma^{m}$(170), and pT7β^{m}, respectively (26). Template DNAs used to measure murine α and ζ globin mRNAs were pT7Mα and pT7Mζ, respectively (1). RNAs were isolated from day 10 yolk sac (1,000 ng), day 12 liver (500 ng), day 12 blood (80 ng), day 14 liver (500 ng), and adult blood (80 ng). Signals were quantitated with a PhosphorImager (Molecular Dynamics).

Immunofluorescent detection of human globin chains. Globin chains were visualized by staining fixed cells with ε-, γ-, or β-globin-specific monoclonal antibodies. Cytocentrifuge smears were fixed in methanol and incubated with appropriate antibodies. A second antibody [goat F(ab′)$_2$ anti-mouse fluorescein isothiocyanate-conjugated immunoglobulin G; Dupont, Wilmington, Del.] that is reactive to the mouse monoclonal antibodies was added to allow color detection of the globin proteins.

RESULTS

Structural analysis of transgenic mice. Previous studies have shown that YAC transgenes frequently have deletions of the 5′ and 3′ sequences, thus requiring detailed structural analysis of the YAC DNA integrated in transgenic mice (30–33). Identification of mice bearing intact β-globin loci is an essential prerequisite to functional studies. The continuity of the β-globin locus within individual YAC copies was determined as described in Materials and Methods and shown in Fig. 2. Four lines had at least one intact 140-kb *Sfi*I fragment and were used in this study. The presence of HS4, which resides upstream of the 5′ *Sfi*I site used in our structural analysis, was confirmed in all lines (Fig. 3). Line A has an intact 140-kb fragment and an additional 120-kb fragment containing a β-globin locus with a deletion 3′ to the δ-globin gene. Thus, line A has two copies of ε-, $^{G}\gamma$-, $^{A}\gamma$-, and δ-globin genes but a single β-globin gene. Line B has a single 140-kb fragment containing the entire β-globin locus. Line C has two intact β-globin loci of 150 and 160 kb. Line D contains an intact β-globin locus on a 135-kb fragment which is missing sequences downstream of the β-globin gene, including 3′ HS1; the deletion in the 135-kb fragment, however, spares the enhancer element (2, 23, 43), which is located 0.4 kb 3′ of the β-globin locus (Fig. 4). Structural rearrangements of some YAC copies (deletions of the 5′ or 3′ end or both ends) are common in β-YAC transgenics (31–33). However, if the β-globin locus itself is intact (i.e., from 5′ HS4 through the β-globin gene enhancer), developmental expression of the globin genes is normal both temporally and spatially and there is position-independent, copy-number-dependent expression of the globin genes (33).

Deletion of the HS3 core element abolishes ε-globin gene expression during embryonic erythropoiesis. Transgenic mice carrying a wild-type β-YAC express human ε-globin mRNA in the yolk sac stage of erythropoiesis. ε-Globin mRNA ranges from 10 to 20% of murine α- plus ζ-globin mRNA (per copy)

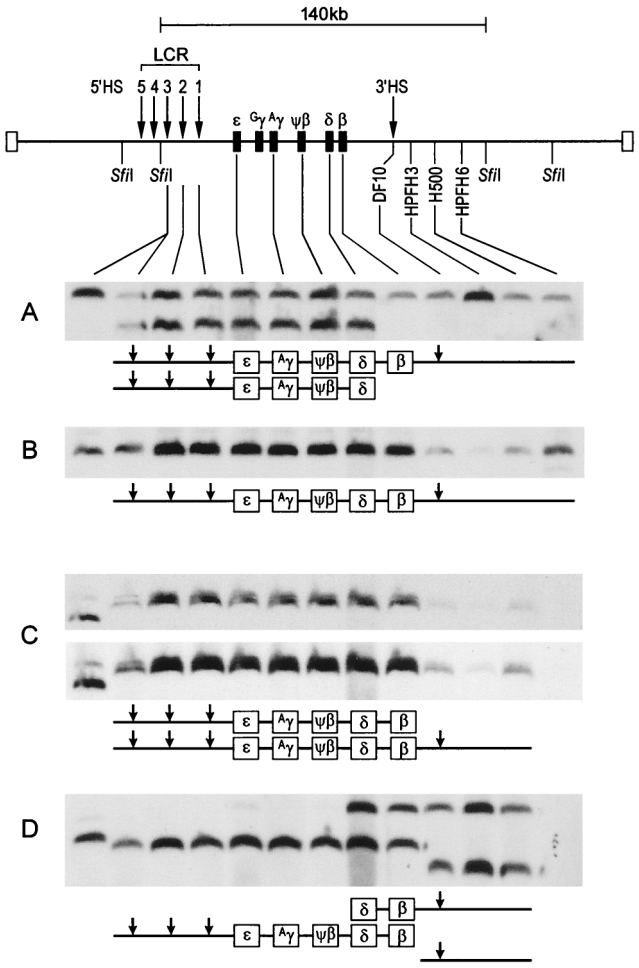

FIG. 2. Structures of the β-YAC globin loci of ΔHS3c β-YAC transgenic mouse lines. The upper part of the figure shows the 140-kb *Sfi*I fragment encompassing most of the β-globin locus from 5′ HS3 to the breakpoint of HPFH6 approximately 53 kb downstream of the β-globin gene. Arrows indicate HSs. Agarose plugs containing high-molecular-weight DNA from liver tissue were isolated from four ΔHS3c β-YAC transgenic lines. The plugs were digested with *Sfi*I, fractionated by PFGE, and subjected to Southern hybridization analyses. The location of each of the probes used in the structural analysis is identified on the map. Schematic representations of the structures of the *Sfi*I fragments are drawn below each autoradiogram. (A) Line A has an intact 140-kb fragment and an additional 120-kb fragment which is deleted from sequences 3′ of the δ-globin gene. (B) Line B has a single 140-kb fragment identified by each of the probes. (C) Line C has a 150-kb fragment containing the intact locus from 5′ HS3 to position H500 (placed between the breakpoints of HPFH3 and HPFH6). A second 160-kb fragment extends from 5′ HS3 to the β-globin gene. This fragment can be seen in the doublets apparent in the lighter exposure of the same autoradiogram in the upper portion of panel C. (D) Line D has a 140-kb fragment containing an intact β-globin locus and two additional fragments of 120 and 170 kb, from both of which most of the β-globin locus is deleted. The first lane in each of the autoradiograms contains control DNA from a mouse erythroleukemia cell line containing a single intact β-YAC, as determined by structural analysis and fluorescent in situ hybridization.

in the yolk sac, and it continues to be synthesized in circulating embryonic erythroblasts. Synthesis peaks in the embryonic erythroblasts at about day 12 of development.

Developmental studies were performed with yolk sac and blood samples from day 10 F_2 embryos and with blood samples from day 12 and 14 F_2 fetuses. Staining of fixed yolk sac or blood preparations with anti-human ε-globin fluorescent monoclonal antibody failed to detect any ε-globin in the primitive erythroblasts of the ΔHS3c transgenic embryos (not shown).

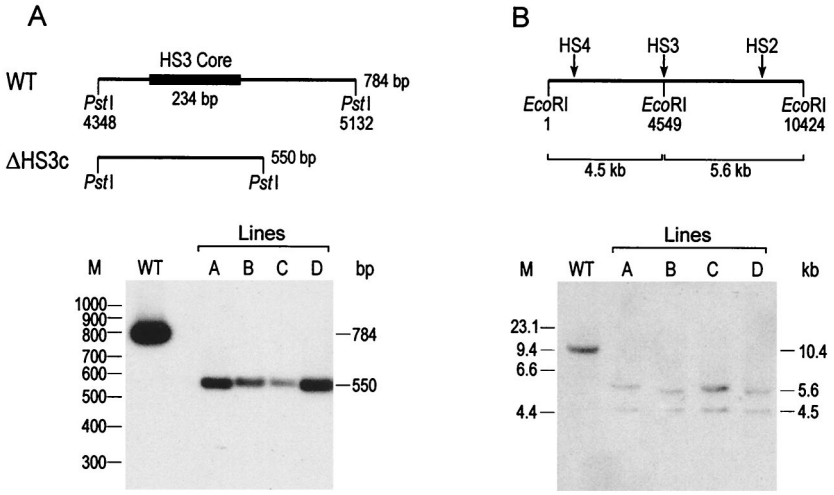

FIG. 3. Southern analysis of the HS3c deletion showing the presence of HS4 in the ΔHS3c β-YAC transgenic lines. (A) A map of the wild-type 784-bp *Pst*I fragment (GeneBank coordinates indicated) and the 550-bp *Pst*I fragment deleted from the HS3c elements is shown above the autoradiograph. All DNA samples were digested with *Pst*I. Lane WT contains DNA from a transgenic mouse line carrying a wild-type β-YAC. Lanes A to D correspond to the four lines of Fig. 2. Each line has the predicted 550-bp fragment which is diagnostic of the HS3c deletion. The probe was the 784-bp *Pst*I HS3 fragment. M, molecular weight. (B) HS2 to -4 reside on a 10.4-kb *Eco*RI fragment (GeneBank coordinates indicated) in the wild-type β-globin locus. As shown in the diagram, the creation of an *Eco*RI site by site-directed mutagenesis and subsequent deletion of the HS3c element results in a 4.5-kb *Eco*RI fragment containing HS4 and a 5.6-kb fragment containing HS2. The autoradiogram shows the results of *Eco*RI digestion of samples from the control (lane WT) and lines A to D. Notice that all lines contain a 5.6- and a 4.5-kb fragment, indicating that HS4 is linked to the HS3c deletion. The probe was the 1.4-kb *Spe*I-*Hin*dIII fragment spanning the HS3c element. M, molecular size (in kilobases).

Total RNA from yolk sac and blood samples from multiple embryos of the same litter were subjected to RNase protection analysis with human ε- and γ-globin and mouse α- and ζ-globin antisense RNA probes. In contrast to the wild-type β-YAC controls, ε-globin mRNA was undetectable in day 10 yolk sac from all four ΔHS3c β-YAC lines (Fig. 5). In the day 12 blood, where peak values of ε-globin mRNA are normally found, ε-globin mRNA was not detected in one of the lines and was 1.5% or less in the other three lines (Fig. 6). These data suggest that the HS3 core element is necessary for ε-globin gene transcription during the embryonic stage of erythropoiesis.

Normal γ-globin gene expression in the embryonic cells of ΔHS3c transgenic mice. In contrast to the severe reduction of ε-globin gene expression, γ-globin gene expression was normal in embryonic erythrocytes. Multiple embryos from the same litter were used for RNase protection assays to minimize experimental error and determine sample variation. As shown in Table 1, all lines displayed, in the day 10 yolk sac, levels of γ-globin mRNA that were similar to those observed in control wild-type β-YAC mice. Similar results were obtained with day 12 fetal blood (Fig. 6), which consisted mostly of nucleated erythrocytes of yolk sac origin. Mean levels of γ-globin mRNA were 71 and 72% of those of the controls on days 10 and 12, respectively, but the difference from levels in the wild-type β-YAC control mice was not statistically significant. Most importantly, there was only a small degree of variation in the per copy levels of γ-globin mRNA between lines; levels of γ-globin mRNA in ΔHS3c embryos varied by 1.5-fold, indicating the absence of position effects. A statistical measure of variability is the coefficient of variation (σ/μ) in the levels of per copy expression between lines. Coefficients of variation smaller than 0.5 in levels of globin gene expression between lines denote a small, statistically insignificant degree of variation (25, 35). As calculated from the data of Table 1, the coefficients of variation in the levels of per copy γ-globin gene expression were 0.16 and 0.26 for day 10 and 12 embryonic erythropoiesis in the ΔHS3c lines and 0.26 and 0.27 for day 10 and 12 embryonic erythropoiesis in the wild-type β-YAC controls. These results

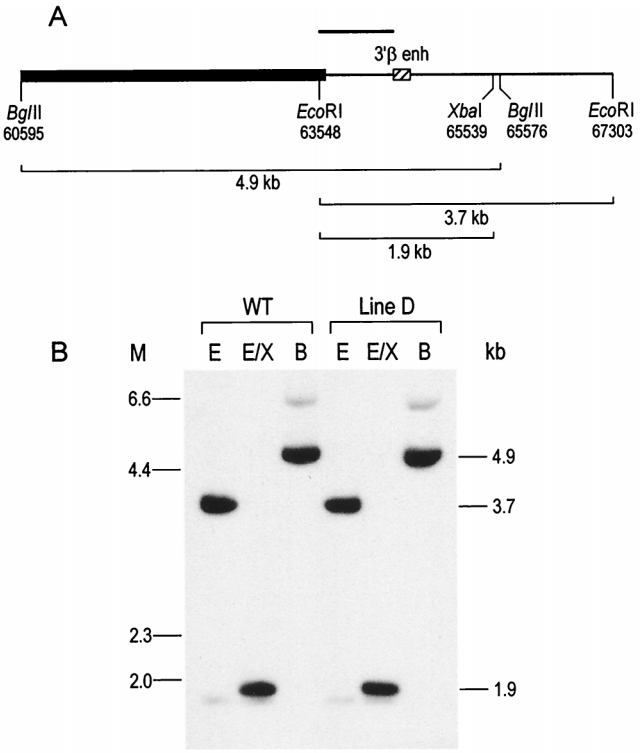

FIG. 4. Detection of the 3′ β enhancer (3′β enh) in line D by Southern analysis. (A) Map of the location of the 260-bp *Pst*I fragment containing the 3′ β enhancer (indicated by the hatched box) relative to the β-globin gene (indicated by the black box). Restriction enzyme sites and GenBank coordinates are shown below the map. The fragment sizes in wild-type DNA indicated below the map are 4.9 kb for *Bgl*II, 3.7 kb for *Eco*RI, and 1.9 kb for *Eco*RI-*Xba*I. (B) Southern analysis of DNAs from the wild-type (WT) β-YAC line and line D digested with the restriction enzymes indicated above each lane as follows: B, *Bgl*II; E, *Eco*RI; and E/X, *Eco*RI-*Xba*I. The probe was a 771-bp *Eco*RI-*Pst*I fragment indicated in panel A as a solid bar. Migration positions of λ*Hin*dIII size markers are indicated to the right of the autoradiogram, and positions of molecular size markers (M) (in kilobases) are indicated to the left.

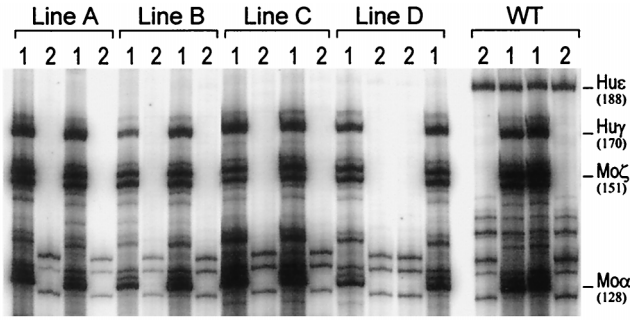

FIG. 5. Expression of the human ε-globin gene in day 10 embryonic yolk sacs from F_2 progeny of β-YAC transgenic lines containing the deletion of the HS3c element. Two littermates were examined from each transgenic line. Lanes 1, RNase protections with antisense probes for human ε- and γ-globin and murine α- and ζ-globin mRNAs; lanes 2, RNase protections with only a human ε-globin mRNA antisense probe in order to unambiguously test for the presence of ε-globin mRNA. Notice the presence of ε-globin mRNA in the control (wild type [WT]) and its complete absence in the lanes of lines A to D. The migrations of the protected fragments are shown to the right of the autoradiogram; the size of each fragment (in bases) is shown in parentheses. Huε, human ε-globin; Huγ, human γ-globin; Moα, mouse α-globin; moζ, mouse ζ-globin.

show that the level of γ-globin gene expression in the embryonic cells of the ΔHS3c mice was nearly normal and that it was not influenced by the position of integration of the transgene.

Deletion of the HS3 core abolishes γ-globin gene expression in definitive erythroid cells. The definitive stage of erythropoiesis begins in the murine fetal liver on day 10.5, and it is characterized by the exclusive transcription of the two adult globins, β-major and β-minor. In transgenic mice carrying a wild-type human β-YAC, the γ-globin genes were active in the fetal liver and the mean γ-globin mRNA level in the livers of the day 12 wild-type β-YAC fetuses was 16.1% ± 6.7% of the murine α- and ζ-globin mRNA levels (Table 1). In contrast, levels of γ-globin mRNA in the day 12 livers of ΔHS3c transgenic fetuses were strikingly reduced and ranged from 1.3 to 2.2% of levels of murine α- and ζ-globin (Fig. 6 and Table 1). In wild-type β-YAC transgenics there is a rapid switch from γ- to β-globin, so that by day 14 the mean level of γ-globin mRNA in fetal liver is 6.4% ± 3.4% of the levels of murine α- and ζ-globin mRNA (Table 1). γ-Globin mRNA was not detectable by the RNase protection assay in the livers of the 14-day-old fetuses of the four ΔHS3c lines (Table 1).

Embryonic erythroblasts contaminate the fetal livers of transgenic mice and contribute to the species of globin mRNAs detected in the fetal liver preparations from early fetuses (reference 38 and unpublished data). It was therefore possible that the low levels of γ-globin mRNA present in the liver RNA samples of the day 12 ΔHS3c fetuses derived from contaminating embryonic erythroblasts. To test this possibility, day 12 fetal liver preparations were stained with anti-γ-globin-chain monoclonal antibodies. As shown in Fig. 7A and B, only embryonic erythroblasts of yolk sac origin, characterized by their large size, their large cytoplasm/nucleus ratio, and their pycnotic nucleus, stained with the anti-γ-globin-chain fluorescent antibody. There was no fluorescent labeling of the definitive erythroblasts other than background staining. These results suggest that the γ-globin mRNA measured in the livers of the day 12 ΔHS3c fetuses derived from embryonic erythroblasts and that there was no detectable γ-globin gene expression in the definitive erythroid cells of fetal liver origin.

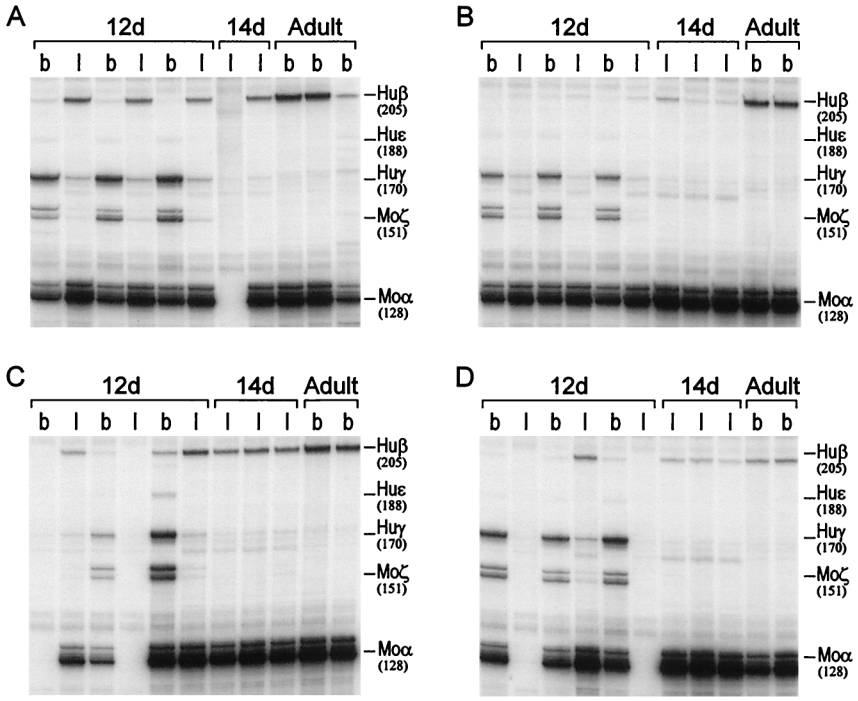

FIG. 6. mRNA analysis of F_2 progeny of β-YAC transgenic mice carrying a deletion of the HS3c element. Total RNA was isolated from day 12 liver and blood, day 14 liver, and adult blood from two or three F_2 littermates from each line and subjected to RNase protection analysis. The migrations of the protected fragments are shown to the right of each autoradiogram; the size of each fragment (in bases) is shown in parentheses. Panels A to D correspond to lines A to D. Lanes b contain blood mRNA; lanes l contain liver RNA. Notice in the day 12 (12d) samples of all lines the normal levels of γ-globin mRNA in the blood (see also Table 1) and the striking decreases in levels of γ-globin mRNA in the day 12 and 14 liver mRNA preparations. Also notice the variation in β-globin mRNA levels in the day 12 or 14 liver samples as well as in the adult blood. See the legend to Fig. 5 for clarification of the abbreviations.

TABLE 1. Human globin mRNA levels per copy of transgene and copy of endogenous murine α-globin gene in ΔHS3c mice and wild-type β-YAC control mice

Line or mouse type	Copy no.	% of murine α- plus ζ-globin mRNA (mean ± SD)								
		ε-Globin mRNA during embryonic erythropoiesis		γ-Globin mRNA during:				β-Globin mRNA during definitive erythropoiesis		
				Embryonic erythropoiesis		Definitive erythropoiesis				
		Day 10 yolk sac	Day 12 blood	Day 10 yolk sac	Day 12 blood	Day 12 liver	Day 14 liver	Day 12 liver	Day 14 liver	Adult blood
A[a]	2	0	1.1 ± 0.2	17.4 ± 2.4	23.9 ± 1.0	2.2 ± 0.5	0	20.6 ± 1.6	13.7 ± 6.4	59.4 ± 5.8
B	3	0	0	12.3 ± 2.9	25.4 ± 1.8	1.9 ± 0.4	0	3.1 ± 0.8	0.8 ± 0.2	3.0 ± 0.5
C	2	0	1.3 ± 0.1	15.7 ± 5.0	11.5 ± 1.0	1.3 ± 0.4	0	11.7 ± 1.2	8.6 ± 0.6	17.1 ± 0.2
D	1	0	1.5 ± 0.3	19.2 ± 5.4	23.9 ± 1.0	2.2 ± 0.5	0	2.0 ± 0.2	2.7 ± 1.3	27.8 ± 0.2
Mean of ΔHS3c line				16.1 ± 2.5	21.2 ± 5.6	1.9 ± 0.4		9.3 ± 7.5	6.4 ± 5.1	26.8 ± 20.8
Wild-type β-YAC control mice		10.2 ± 1.4	22.4 ± 6.5	22.7 ± 5.8	29.5 ± 7.9	16.1 ± 6.7	6.4 ± 3.4	38.9 ± 12	58.0 ± 26	117.0 ± 41

[a] Expression levels are based on two copies of the ε-, ^{G}γ-, and ^{A}γ-globin genes but a single copy of the β-globin gene (see Fig. 2).

β-Globin gene expression in ΔHS3c β-YAC transgenic mice is decreased and position dependent. In wild-type β-YAC mice, β-globin gene expression is similar to that of the endogenous murine genes and there is relatively small variation in the levels of per copy β-globin gene expression among lines. β-Globin gene expression is copy number dependent, indicating that the genes of the β-YAC are protected from position effects (31–33). The small degree of variation in levels of β-globin gene expression in the wild-type β-YAC mice is reflected in the small coefficient of variation (0.35) of the control lines, shown in Table 1. β-Globin gene expression in the ΔHS3c mice was significantly lower than that in control mice, and per copy levels of β-globin gene expression displayed striking variation. The per copy levels of β-globin mRNA varied, among the four lines, 10- and 17-fold in the day 12 and 14 fetal liver definitive erythroblasts and 19.8-fold in adult erythrocytes (Table 1), indicating that β-globin gene expression is strongly influenced by the position of integration of the transgene. Coefficients of variation for the levels of per copy β-globin gene expression for the day 12 and 14 fetuses and the adult mice were 0.8, 0.79, and 0.77, respectively. The presence of strong position effects was also reflected in the striking heterogeneity in the staining of the definitive erythroblasts of the fetal liver preparations with the anti-β-globin-chain fluorescent antibody (Fig. 7C and 7D).

DISCUSSION

Developmental specificity of the interaction between the core of HS3 and globin genes. Our results show that deletion of the core sequence of DNase I-hypersensitive site 3 of the LCR results in the absence of ε-globin gene expression in day 10 embryonic cells and in the absence of γ-globin gene expression in the cells of definitive erythropoiesis in the fetal liver. These results provide direct evidence that there is developmental specificity in the interactions between the LCR and the globin genes: in the presence of an otherwise intact LCR, the core of HS3 is necessary for the activation of the ε-globin gene in embryonic cells and for activation of the γ-globin gene in definitive cells.

That the HSs of the LCR may display developmental specificities was first shown by Fraser et al. in experiments with transgenic mice (14). Those authors produced a series of recombinant constructs in which LCR sequences containing DNase I-HS1, -2, -3, and -4 were linked to an ~36-kb cosmid containing the region of the β-globin locus from the ^{G}γ-globin gene to the β-globin gene (14). Transgenic mice carrying the HS2-^{G}γ to -β cosmid expressed the γ-globin gene in the yolk sac and the β-globin gene in the adult cells, suggesting that HS2 interacts equally well with the γ- and β-globin genes. In contrast, the mice carrying the HS3-^{G}γ to -β cosmid were characterized by high γ-globin expression in embryos and fetuses and low β-globin expression in adults, indicating a preferential interaction of HS3 with the γ-globin gene. The opposite phenotype was observed in HS4-^{G}γ to -β transgenics (14). Developmental specificity of HS3 was also demonstrated in a study of transgenic mice carrying HS3-γβ and HS2-γβ constructs that showed a qualitative difference between HS2 and HS3 with respect to γ-globin gene expression (25). Our results support these previous conclusions and, further, provide evidence that specific sequences of the LCR interact with specific globin genes at specific stages of development.

Bungert et al. (3) have produced transgenic mice carrying either a deletion of HS3c or a replacement of HS3c with HS4c in the context of a 150-kb β-YAC. One ΔHS3c line showed nearly normal γ-globin gene expression and the near absence of ε-globin gene expression in the yolk sac cells. Two lines with substitutions of HS4c for HS3c had a significant reduction of ε-globin gene expression, as would be expected if HS3c is necessary for activation of the ε-globin gene in embryonic cells.

The LCR may change conformations during development. Studies of γ- and β-globin primary transcripts in fetal erythroid cells of transgenic mice carrying a normal β-globin locus have shown that the LCR interacts with only one gene at any given time and that it switches back and forth between the two genes in a flip-flop type of mechanism (46). Similar results have been obtained by Fraser et al. (12a) with embryonic cells in which both the ε- and the γ-globin gene are transcribed from a single locus. Such oscillations of the interactions of the LCR with the ε- and the γ-globin genes should occur in the embryonic cells of transgenic mice carrying the ΔHS3c β-YAC. If the γ-globin genes of the embryonic cells interacted with the mutant HS3 whose core is deleted, these γ-globin genes should have been transcriptionally inactive. Since γ-globin gene expression was normal in the embryonic cells of the ΔHS3c mice, an HS of the LCR other than HS3 should have engaged the γ-globin gene and activated its promoter. These results suggest that different HSs of the LCR interact with the γ- or the ε-globin gene in an embryonic cell. On the basis of the transcriptional behavior of the γ-globin genes in embryonic cells (normal expres-

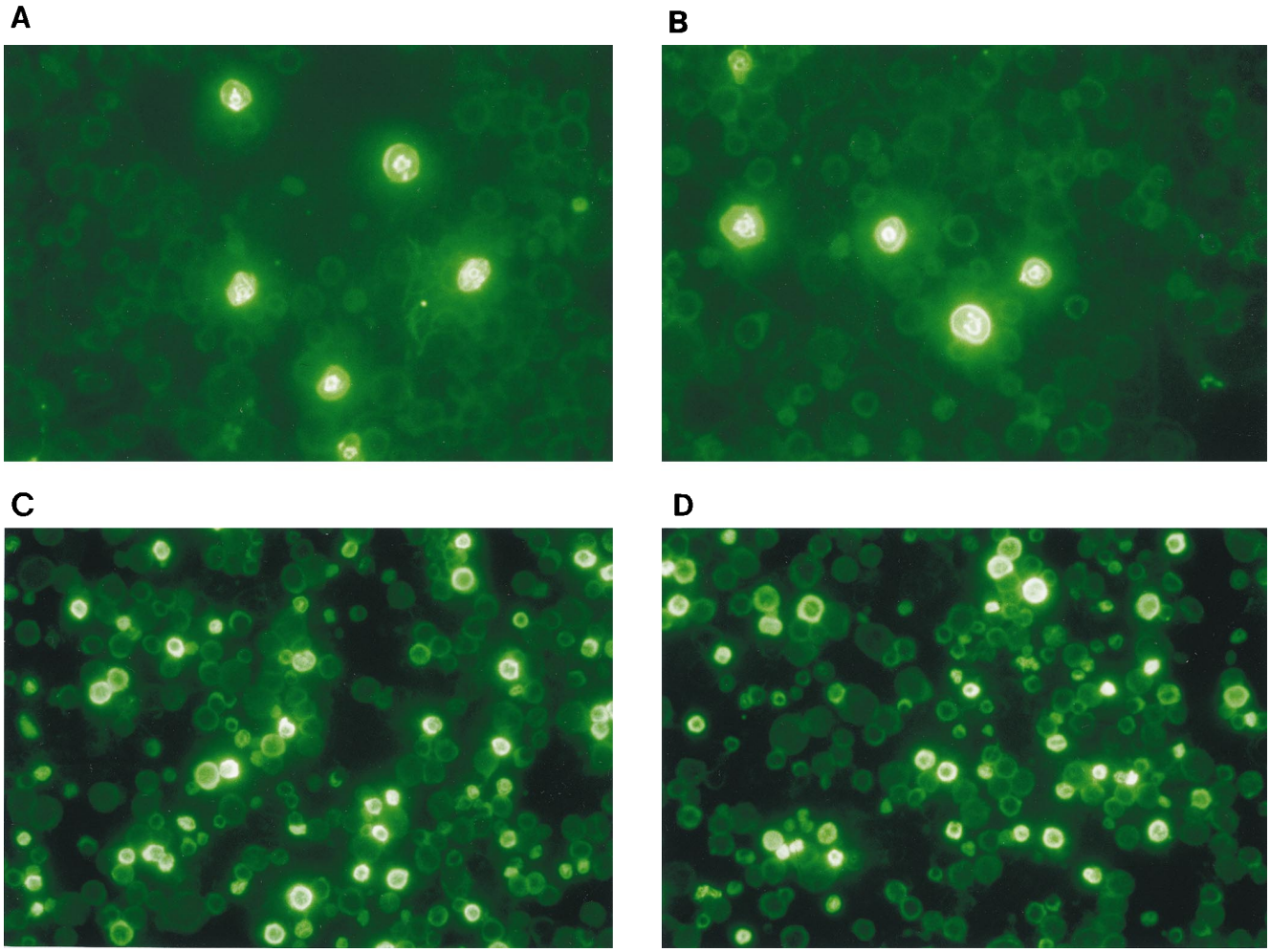

FIG. 7. Staining with fluoroscein isothiocyanate conjugated anti-human globin-chain antibodies of day 12 fetal liver preparations of F_2 transgenic mice carrying the HS3c deletion. (A and B) Staining with anti-γ-globin-chain antibodies. Notice that only the embryonic erythroblasts, recognized by their large size and high cytoplasm/nucleus ratio, are stained. (C and D) Staining with the anti-β-globin-chain antibodies. Notice the heterogeneity of β-globin-chain synthesis among the erythroblasts of definitive erythropoiesis; several nonstained embryonic erythrocytes can be seen in the background of panels C and D.

sion) and in fetal cells (no expression), we can also conclude that different HSs of the LCR interact with the γ-globin genes of the embryonic cells or with the γ-globin genes of the fetal cells.

The prevailing hypothesis of the structure-function relationship of the LCR is that LCR sequences interact with various constitutive and erythroid-lineage-specific transcriptional factors in a way that leads to formation of a complex (holocomplex [46]). Perhaps this complex attains a specific conformation which is optimal for interaction of the LCR with a globin gene. The strikingly different phenotypes of γ-globin gene expression in embryonic and fetal cells raise the possibility that the LCR attains different conformations in order to interact with the γ-globin genes of embryonic cells or with the γ-globin genes of definitive cells. The conformation of the LCR may change as the transcriptional milieu of the erythroid cells changes during the course of development.

Role of the core of HS3 in the opening of the β-globin locus chromatin domain in embryonic and in definitive erythroid cells. Although β-globin mRNA was present in the ΔHS3c adult mice, β-globin mRNA levels varied by 19.8-fold among lines, indicating that β-globin gene expression is strongly influenced by the position of integration of the transgene. These results support previous suggestions that the core of HS3 is required for opening of the globin chromatin domain in cells of definitive (liver-stage) erythropoiesis (6). In contrast to the position-sensitive β-globin gene expression in definitive erythroid cells, there was minimal variation in levels of γ-globin mRNA among the ΔHS3c lines, indicating that γ-globin gene expression in the embryonic cells of the ΔHS3c transgenic mice is not influenced by the position of the integration of the transgene. These results suggest that, in contrast to the adult cells, the core of HS3 is not an important contributor to the function of the LCR, which opens the globin locus domain in embryonic cells. Apparently, in the ΔHS3c mice the domain-opening function of the LCR is conducted by other DNase I HSs.

The phenotypes of deletions that remove the core of HS3 as well as the sequences flanking the core. Peterson et al. (32) have deleted 2.3 kb of HS3 (including the HS3 core) in the context of a 248-kb β-YAC. Transgenic mice carrying these ΔHS3 β-YACs displayed, in embryonic cells, about a threefold reduction in ε-globin gene expression compared to that of controls but no reduction of γ-globin gene expression in the fetal cells. Hug et al. (19) deleted 2.3 kb of murine HS3 through homologous recombination in embryonic stem cells

and analyzed murine globin gene expression in chimeric mice; there was only a small (about 20%) reduction in expression of the murine globin genes in embryonic or in definitive erythropoiesis. It thus appears that the specific effects on ε- and γ-globin gene expression produced by the HS3 core deletions are not observed when the sequences flanking the core are also deleted. One way of reconciling these results is to assume that the core of HS3 and the HS3 flanking sequences possess different, but complementary, functions. The sequences flanking the core of HS3 may function by engaging a globin gene and positioning it in a way that allows optimal interaction of the gene with the HS3 core element; the HS3 core element, on the other hand, may interact with the transcriptional complex of the gene, thus activating globin gene expression. When the core element is deleted, as in the ΔHS3c β-YAC, the HS3 flanking region still interacts with the globin gene but activation of transcription does not occur. When the whole HS3 is deleted, another HS interacts with the γ-globin gene and there is minimal effect on gene expression. Redundancy of the functions of HSs is supported by several observations (8, 19, 32), and there is evidence from various experiments suggesting that the flanking sequences of HSs have a role in HS function (21, 24, 29).

The HS3 core and embryonic expression of the γ-globin gene. Several species have genes which are orthologous to the γ-globin genes of primates, but they are expressed only in embryonic cells. The βh1 gene of the mouse is such an example. The expression of the γ-globin genes of primates was initially limited to the embryonic stage of erythropoiesis until the so-called fetal recruitment of the γ-globin genes; i.e., the expression of γ-globin genes in the definitive cells of the fetal liver stage of erythropoiesis occurred about 30 to 50 million years ago (17). As we show here, when the core of HS3 is deleted, the γ-globin gene becomes an embryonic gene, i.e., it is expressed exclusively in embryonic cells. This reversion of the γ-globin gene to its ancestral developmental pattern raises the possibility that the sequences of the core of HS3 may have contributed to the recruitment of its expression in fetal erythroid cells. Mutations that accumulated, during the evolution of primates, either in the γ-globin gene promoter, in the HS3 core sequence, or in both may have created the protein binding site(s) which allows the interaction between the core of HS3 and the γ-globin gene to occur in the cells of fetal liver erythropoiesis.

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