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Effect of deletion of 5′HS3 or 5′HS2 of the human β-globin locus control region on the developmental regulation of globin gene expression in β-globin locus yeast artificial chromosome transgenic mice

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ABSTRACT **To analyze the function of the 5′ DNase I hypersensitive sites (HSs) of the locus control region (LCR) on β-like globin gene expression, a 2.3-kb deletion of 5′HS3 or a 1.9-kb deletion of 5′HS2 was recombined into a β-globin locus yeast artificial chromosome, and transgenic mice were produced. Deletion of 5′HS3 resulted in a significant decrease of ε-globin gene expression and an increase of γ-globin gene expression in embryonic cells. Deletion of 5′HS2 resulted in only a small decrease in expression of ε-, γ-, and β-globin mRNA at all stages of development. Neither deletion affected the temporal pattern of globin gene switching. These results suggest that the LCR contains functionally redundant elements and that LCR complex formation does not require the presence of all DNase I hypersensitive sites. The phenotype of the 5′HS3 deletion suggests that individual HSs may influence the interaction of the LCR with specific globin gene promoters during the course of ontogeny.**

The human β-globin locus spans approximately 82 kb of chromosome 11. The locus consists of a powerful upstream control element, the locus control region (LCR), and five functional β-like globin genes arrayed 5′ to 3′ in the order in which they are expressed during development. Six DNase I hypersensitive sites (HSs) flank the globin genes (1, 2). One site is located approximately 20 kb downstream from the β-globin gene (3′HS1), and five sites are located 6–22 kb upstream of the ε-globin gene (5′HS1 to 5′HS5). 5′HS5 is constitutive, while 5′HS1–4 are erythroid-specific (1–5). The LCR activates the β-globin locus chromosomal domain, insulates the globin genes from the effects of surrounding chromatin, restricts globin gene expression to cells of the erythroid lineage, and acts as a powerful enhancer directing high levels of globin production in erythroid cells (refs. 4 and 6; for review, see refs. 7 and 8).

Most functional analyses of the DNase I hypersensitive sites of the LCR have been carried out in transgenic mice using simple constructs linking an individual HS region to a single human globin gene or to the $^{G}\gamma$-β genes in a cosmid (9–25). However, these constructs only test the ability of the individual sites to function as enhancers of linked transgene expression, and they do not reveal their role in the context of the native LCR. Testing the contribution of each HS to LCR function requires analysis of the effects of individual HS mutations in the context of the whole β-globin locus. Synthesizing constructs of the 82-kb locus containing these mutations is technically difficult. We previously reported that the analysis of the regulation of the β-like globin genes is greatly facilitated using β-globin locus yeast artificial chromosomes (β-YACs) (26, 27).

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In this paper, we examine the effects of deletion of 5′HS3 or 5′HS2 of the β-globin locus YAC on human β-like globin gene expression. We demonstrate that when 5′HS3 is deleted, ε-globin gene expression is decreased in the embryonic yolk sac, while deletion of 5′HS2 results in a minor, but statistically significant, decrease in ε-, γ-, or β-globin gene expression. These results indicate that there is functional redundancy among the HSs and that the formation of a LCR complex does not require the presence of all five HSs. The effects of the 5′HS3 deletion on ε-globin gene expression suggest that specific interactions between the HSs and the globin genes underlie the activation of globin genes during specific stages of development.

MATERIALS AND METHODS

Deletion of LCR 5′HS3 in the β-YAC. Plasmid pIII (gift from Dr. Qiliang Li) contains a *Hpa*I–*Kpn*I fragment encompassing 5′HS3 (GenBank coordinates 3375–7764). Plasmid pIII DNA was digested with restriction enzymes *Eco*RI and *Cla*I; the 3′ overhangs were made blunt-ended with *Escherichia coli* DNA polymerase I Klenow fragment, and the treated plasmid DNA was religated to remove a *Hin*dIII restriction enzyme site in the multiple cloning site. The resultant plasmid was digested with *Spe*I and *Hin*dIII, the ends were made blunt, and the DNA was religated to remove 2320 bp of 5′HS3 (GenBank coordinates 3838–6158; see Fig. 1). A *Kpn*I–*Xba*I fragment containing the 5′HS3 deletion and flanking sequences was subcloned into the yeast integrating plasmid (YIP) vector pRS406 (Stratagene) to produce pRS406 ΔHS3.

The 5′HS3 deletion was recombined into the β-YAC by the "pop-in, pop-out" method of homologous recombination in yeast containing the YAC (27). pRS406 ΔHS3 was linearized with *Sph*I (GenBank coordinate 7327), which cuts asymmetrically relative to the deletion breakpoints and gives recombination intervals of 1532 and 437 bp. Selection and identification of yeast isolates containing correct YIP insertions into the β-YAC and selection and structural confirmation of proper YIP excisions were performed as described previously (27). The ΔHS3 β-YAC was purified and microinjected into fertilized mouse eggs as described previously (26–29).

Deletion of LCR 5′HS2 in the β-YAC. Plasmid pII (gift from Dr. Qiliang Li) contains a *Kpn*I-*Hpa*I fragment encompassing 5′HS2 (GenBank coordinates 7764–11,976). A *Kpn*I-*Cla*I fragment containing this region was subcloned into the YIP vector pRS406 (Stratagene). Plasmid DNA of this construct was

Abbreviations: LCR, locus control region; YAC, yeast artificial chromosome; HS, hypersensitive site; YIP, yeast integrating plasmid.

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digested with restriction enzymes *Cla*I and *Bam*HI; the 3′ overhangs were made blunt-ended with Klenow fragment, and the DNA was religated to remove a *Hin*dIII restriction enzyme site. The resultant plasmid was digested with *Hin*dIII and religated to remove 1942 bp of 5′HS2 (GenBank coordinates 8486–10,410; see Fig. 1) to produce pRS406 ΔHS2.

The 5′HS2 deletion was recombined into the β-YAC as described above. pRS406 ΔHS2 was linearized with *Sph*I (GenBank coordinate 11,282), which cuts asymmetrically relative to the deletion breakpoints and gives recombination intervals of 872 and 694 bp. Yeast isolates containing correct YIP insertions and excisions were screened as described above; the ΔHS2 β-YAC was purified and microinjected as described above.

Structural Analysis of ΔHS3 and ΔHS2 β-YACs in Transgenic Mice. Our diagnostic test for intact β-globin loci is the presence, on Southern blots of pulsed-field gels, of a 140-kb *Sfi*I fragment that encompasses the locus from 5′HS3 to approximately 70 kb downstream of the β-globin gene (ref. 27; see Fig. 2*A*). Single cell suspensions prepared from the livers of F_1 transgenic mice were used to prepare agarose blocks containing high mass genomic DNA. These blocks were used for pulsed-field gel electrophoresis Southern blot analysis of YAC structures as described by Peterson *et al.* (27). The variation in *Sfi*I fragment size results from a deletion of one or both of the *Sfi*I sites flanking the 140-kb fragment. *Sfi*I sites in the mouse genome juxtaposed near the 5′ and/or 3′ ends of the transgene produce altered size transgenes that still contain the essential sequences of the β-globin locus. Other Southern blot analyses demonstrated that 5′HS4 was present in the ΔHS3 β-YAC and the ΔHS2 β-YAC lines (data not shown). Copy numbers of all lines were confirmed by a standard method (ref. 26; data not shown).

Measurement of Globin mRNA Synthesis. Total RNA isolation and RNase protection analysis were performed as described (26, 27, 30).

RESULTS

Deletion of 5′HS3 Decreases ε-Globin Gene Expression. A deletion of 2320 bp encompassing 5′HS3 of the LCR (Fig. 1) was introduced into a β-YAC, which was used for production of transgenic mice. Of 110 pups, 14 were transgenic (13%) as judged by slot-blot analysis of tail DNA using an $^{A}\gamma$ gene probe. Two of the lines with intact β-globin loci were used for functional studies. The structural analysis of the integrated transgenes is summarized in the legend of Fig. 2 *A–C*.

In wild-type β-YAC transgenic mouse lines, human ε- and γ-globin mRNA are detected in the yolk sac and blood of embryos as early as 8 days postconception and persist in the blood of developing fetuses through day 14 of development (26, 27, 31). We used RNase protection analysis to determine if deletion of 5′HS3 affected ε-globin expression during the embryonic stage of development. Founders of both ΔHS3 β-YAC lines were bred for F_1 progeny, and F_1 animals from each line were mated to produce F_2 fetuses. Total RNA was isolated from embryonic yolk sacs 10 days postconception or from fetal blood 12 days postconception (which has circulating yolk sac-derived embryonic red cells) and subjected to RNase protection analysis using human ε and mouse ζ and α as probes. Fig. 3 *A* and *B* shows that only low levels of ε-globin mRNA were observed in 10-day yolk sac or 12-day blood for either ΔHS3 β-YAC line. In contrast, similar samples from wild-type β-YAC lines displayed normal levels of ε-globin gene expression (lanes indicated by WT). Fig. 4*A* shows that ε mRNA levels, expressed on a per copy basis, relative to mouse α and ζ mRNA expression, are decreased about an average of 2.8-fold in the 10-day yolk sac and 3.8-fold in 12-day blood.

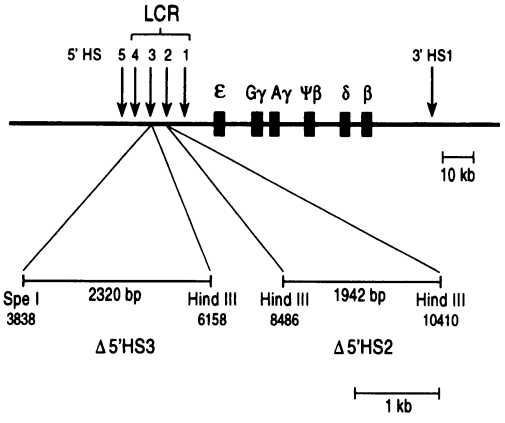

FIG. 1. Location and extent of 5′HS3 and 5′HS2 deletions in the β-YAC LCR.

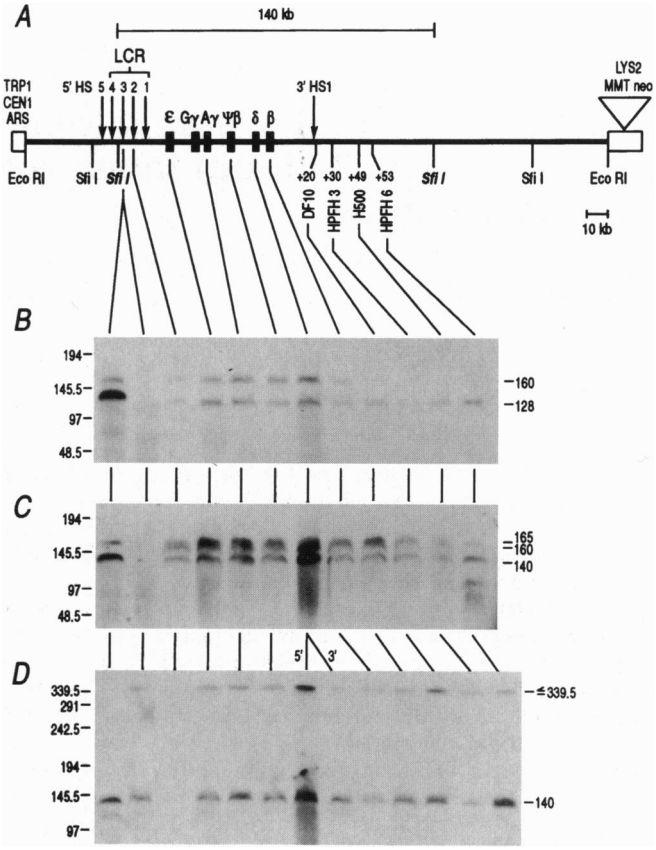

FIG. 2. Structure of the β-globin locus within ΔHS3 and ΔHS2 β-YAC transgenic lines. (*A*) Diagram of the human β-globin locus yeast artificial chromosome (β-YAC). The diagnostic 140-kb *Sfi*I fragment encompassing most of the locus is indicated above the β-YAC map. *Sfi*I digestion, pulsed-field gel electrophoresis, and Southern blot hybridization of the individual lanes were performed as described in *Materials and Methods*. The lines between the map and the autoradiograms show the location of the probes used in the structural analysis for each lane; the names of the probes are shown above or below the map. See ref. 27 for probe template fragments utilized and sources. (*B*) ΔHS3 line 1 contains two intact loci, except for the 5′HS3 deletion, 128 and 160 kb, respectively. (*C*) ΔHS3 line 2 contains three intact loci, 140, 160, and 165 kb (the 160 and 165 kb have very similar mobilities), respectively. (*D*) ΔHS2 line 1 contains copy sizes of 140 and 339 kb; both contain sequences from 5′HS3 to HPFH 6, except for 5′HS2. For this line, two δ gene region probes were utilized, indicated as 5′ and 3′. For panels *B*, *C*, and *D*, the locations of λ pulsed-field gel electrophoresis molecular weight markers are shown at the left (in kb), and the sizes of the *Sfi*I β-globin locus fragments are shown at the right of each autoradiogram.

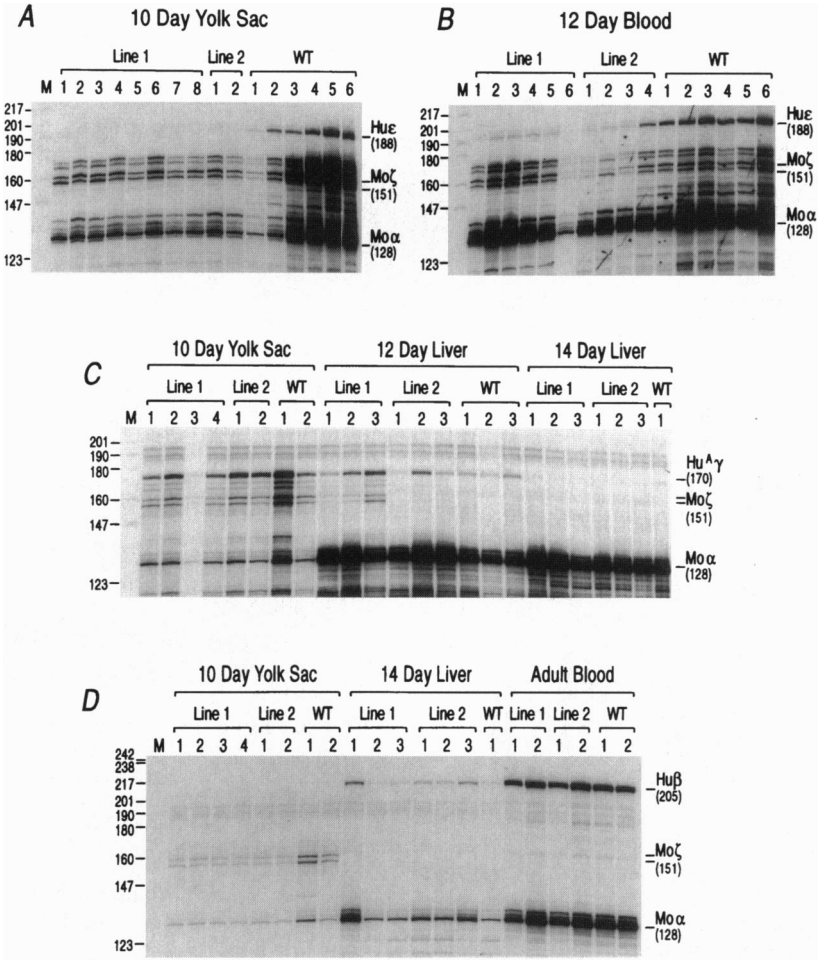

FIG. 3. Expression of human globin transgenes during development in ΔHS3 β-YAC lines. Human ε-globin gene expression is shown in 10-day yolk sac (*A*) or 12-day blood (*B*). Human γ-globin gene expression is shown in 10-day yolk sac, 12-day liver, or 14-day liver (*C*). Human β-globin gene expression is shown in 10-day yolk sac, 14-day liver, and adult blood (*D*). Total RNA was isolated from yolk sac, liver, or blood at the indicated days and subjected to RNase protection analysis as described in *Materials and Methods* using antisense RNA probes. The location of the protected fragments is shown at the right of each autoradiogram; the size of each fragment is shown in parentheses: Human ε, Huε; mouse ζ, Moζ; mouse α, Moα; human ^{A}γ, Hu^{A}γ; human β, Huβ. The ΔHS3 and wild-type (WT) lines are indicated above each autoradiogram, as well the number of each individual within the line. The location of pBR322 *Msp*I-digest molecular weight markers is shown at the left of each autoradiogram.

The human γ-globin genes are expressed in the embryonic yolk sac as well as in the fetal liver in wild-type β-YAC transgenics (26, 27, 31). Fig. 3*C* shows that γ-globin mRNA levels in the 10-day yolk sacs of ΔHS3 β-YAC mice from both lines appear to be similar to those found in wild-type β-YAC lines. However, when expression is calculated on a per copy basis relative to mouse α and ζ expression, γ mRNA is actually increased an average of 1.8-fold compared with levels in wild-type β-YAC lines (Fig. 4*B*). Thus, a reciprocal effect on γ-globin and ε-globin transcription is observed when 5′HS3 is deleted.

5′HS3 Is not Required for γ- or β-Globin Gene Expression in Definitive Erythropoiesis. In 12- or 14-day fetal liver, γ-globin mRNA expression is detected at levels comparable with wild-type for both ΔHS3 β-YAC lines (Figs. 3*C* and 4*B*). Thus, deletion of 5′HS3 has no effect on fetal stage-specific γ-globin gene expression. Similarly, β-globin mRNA expression is detected in increasing amounts relative to mouse α in 14-day fetal liver through the adult stage of development. The observed pattern parallels that found in wild-type β-YAC lines, and the levels appear similar between mutant and wild-type lines. Deletion of 5′HS3 produces a small reduction in the per copy expression of β relative to mouse α in ΔHS3 β-YAC lines compared with wild-type β-YAC lines in day 12 and 14 fetal liver (an average of 1.6-fold at 12 days and 1.4-fold at 14 days; Fig. 4*C*) but does not affect β-globin gene expression in the adult.

Deletion of 5′HS2 Decreases Globin Gene Expression at all Stages of Development. Six of 131 pups produced with the β-YAC containing the 1942-bp deletion of 5′HS2 (Fig. 1) were transgenic. Of the six transgenics, one had an intact β-globin locus and was used to establish a line for functional studies. The structural analysis of this transgene is detailed in the legend of Fig. 2 *A* and *D*. Fig. 5 *A* and *B* show that the human globin mRNA levels from the ΔHS2 β-YAC line are reduced at all stages of development compared with controls. Deletion of 5′HS2 reduces ε gene expression an average of 1.8-fold and γ gene expression 1.5–2 fold. β gene expression is reduced about 2-fold (Fig. 6). Like the deletion of 5′HS3, deletion of 5′HS2 does not affect the temporal pattern of globin gene switching.

DISCUSSION

The main tool for studying LCR function has been the analysis of human globin gene expression in transgenic mice carrying simple constructs linking a single DNase I hypersensitive site of the LCR to one or more globin genes. A considerable amount of information has been gathered from these studies, but, as our results show, this information does not reflect the

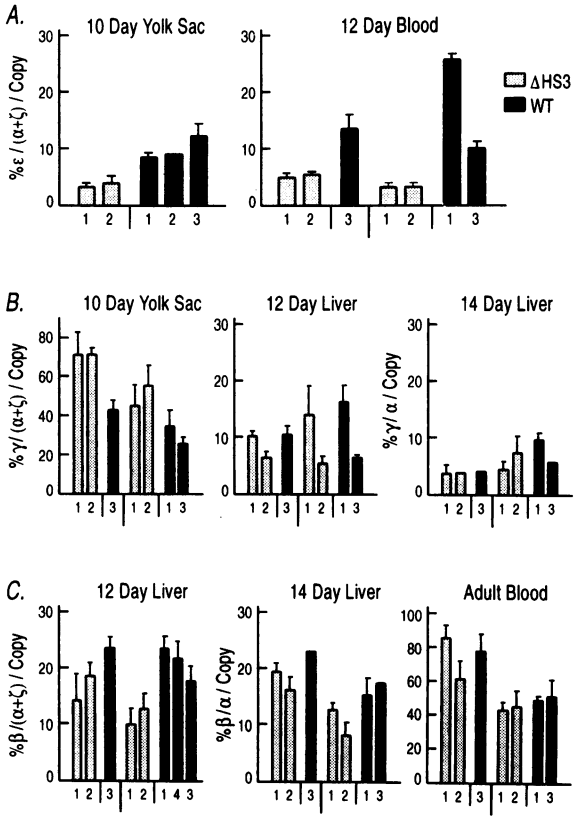

FIG. 4. Human globin mRNA levels in ΔHS3 and wild-type (WT) β-YAC transgenic lines. mRNA levels were quantitated from RNase protection analyses of RNA derived from the indicated tissues on the indicated days by phosphorimaging. Human ε, ^{A}γ, and β mRNA levels were calculated per transgene copy and are expressed as a percentage of endogenous mouse α plus ζ mRNA levels, also corrected for copy number. The average and standard deviation for all individuals within a line are plotted on the *x* axis. Line numbers are indicated on the *x* axis. (*A*) Human ε-globin mRNA expression. (*B*) Human γ-globin mRNA expression. (*C*) Human β-globin mRNA expression.

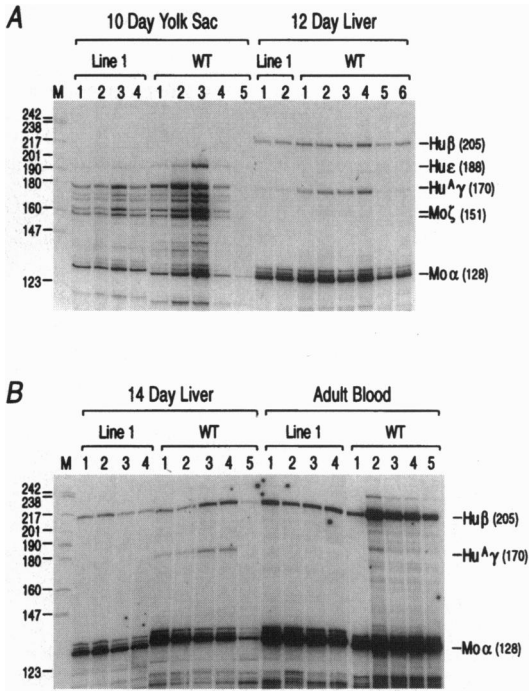

FIG. 5. Expression of human globin transgenes during development in a ΔHS2 β-YAC line. The location of human ε-, γ-, and β- and mouse α- and ζ-globin protected fragments are indicated to the right of each autoradiogram. Other figure details are as described in the legend to Fig. 3. (*A*) 10-day yolk sac and 12-day liver. (*B*) 14-day liver and adult blood.

function of the HSs in the context of the whole β-globin locus. Previous reports indicate that 5′HS2 is a powerful, and possibly the major, enhancer within the LCR complex and that it is necessary for the function of all the globin genes at all stages of development (10, 21). Our work demonstrates that deletion of this DNase I hypersensitive site produces only minor reductions in globin gene expression. 5′HS3 was shown to be the most active site during the embryonic stage of development and was the only site capable of directing high level expression of the γ genes during fetal hematopoiesis (21, 24). We find that deletion of 5′HS3 does not produce the predicted absence or decrease in γ-globin gene expression; instead we observe a specific decrease in ε-globin gene expression with a concomitant slight increase in γ-globin gene expression. Our results show that studies of LCR function need to be done in the context of an intact β-globin locus and that YACs provide the proper tool for the analysis of LCR structure-function relationships.

The current model of LCR function assumes that the individual HSs of the LCR interact with each other to form a holocomplex, which in turn interacts with the individual globin genes by a looping mechanism (21, 32). How this is achieved remains a matter of speculation. In general, LCR structure-function relationships have been difficult to discern due to the large size of the LCR and the β-globin locus. Studies such as those described here will allow the eventual delineation of these relationships. Questions such as the structural requirements for the formation of the LCR complex, the minimum number or combinations of HSs that are essential for LCR holocomplex formation, and the functional significance of the order in which the HSs are arrayed within the LCR can be addressed using YAC technology. Comparison of human and goat LCR sequences has provided evidence that the overall spatial organization of the LCR has been preserved during evolution, in spite of the fact that the structure of the flanking sequences has been changed and different repetitive elements have been inserted between the HSs (33). The functional relevance of the spatial organization of the LCR can be directly tested in the β-globin locus YAC by examining the effects of altering the HS spacing on LCR function.

The fact that deletion of 5′HS3 specifically represses ε-globin gene expression suggests that sequences of the LCR are directly or indirectly involved in globin gene switching. Developmental stage specificity of the interaction between sequences of the LCR holocomplex and sequences of the globin genes may be achieved by the presence of transcription factors that increase the probability of interaction of an HS with a specific globin gene promoter. Alternately, intra-LCR interactions between the HSs may result in formation of specific embryonic, fetal, or adult conformations of the LCR holocomplex, and these stage-specific conformations facilitate the interaction of the LCR with specific globin genes. Perhaps switching is regulated through a composite of changes in LCR conformation and changes in the availability of trans-acting factors that enhance the interaction between the LCR and specific globin gene promoters at each stage of development.

Recently, Bungert *et al.* (34) reported that deletion of 280 bp of the 5′HS4 core element or 225 bp of the 5′HS3 core element in the context of a β-YAC resulted in catastrophic disruption of globin gene expression at all stages of ontogeny. These results are in contrast to our data and that of Fiering *et al.* (35), showing that entire HS deletions produce only modest effects on globin gene expression. The data of Bungert *et al.* (34), if confirmed, will indicate that HS core region deletions function

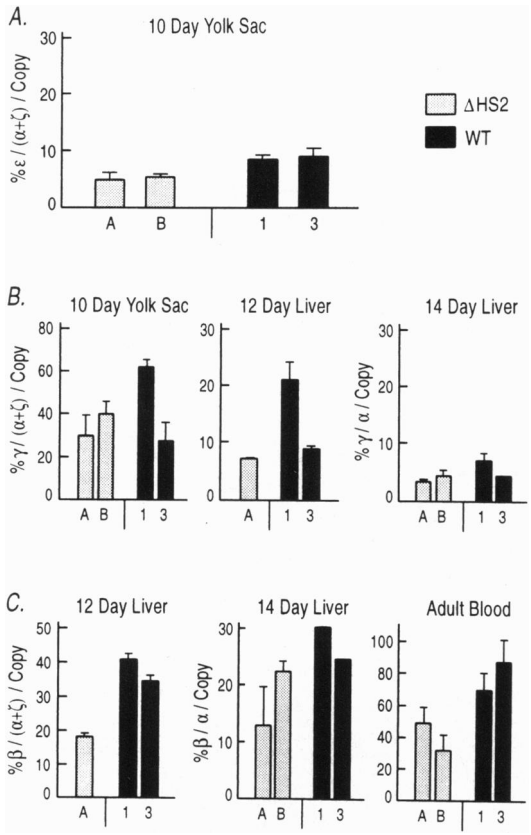

FIG. 6. Human globin mRNA levels in ΔHS2 and wild-type (WT) β-YAC transgenic lines. mRNA quantitation was performed as described in the legend to Fig. 4. On the *x* axis, A and B represent independent experiments for ΔHS2 line 1; 1 and 3 indicate numbers of wild-type lines. (*A*) Human ε-globin mRNA expression. (*B*) Human γ-globin mRNA expression. (*C*) Human β-globin mRNA expression.

as dominant negative mutations that inactivate the function of the LCR. Perhaps the HS core elements interact with one another to form the active center of the LCR, while the sequences flanking the HS cores mediate the folded structure of the LCR holocomplex. Under this hypothesis, deletion of only one HS core sequence will not affect the conformation of the LCR holocomplex but will disrupt the function of the active center resulting in catastrophic effects on globin gene expression. In contrast, deletion of an entire HS (core and flanking regions) will allow the LCR to adapt an alternate holocomplex conformation with a modified active center that functions less efficiently.

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