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Deletion of the human β-globin LCR 5'HS4 or 5'HS1 differentially affects β-like globin gene expression in β-YAC transgenic mice

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Abstract

A 213 Kb human β-globin locus yeast artificial chromosome (β-YAC) was modified by homologous recombination to delete 2.9 Kb of cross-species conserved sequence similarity encompassing the LCR 5' hypersensitive site (HS) 4 (Δ5'HS4 β-YAC). In three transgenic mouse lines, completion of the γ- to β-globin switch during definitive erythropoiesis was delayed relative to wild-type β-YAC mice. In addition, quantitative per-copy human β-like globin mRNA levels were similar to wild-type β-YAC transgenic lines, although β-globin gene expression was slightly decreased in the day 12 fetal liver of Δ5'HS4 β-YAC mice. A 0.8 Kb 5'HS1 fragment was similarly deleted in the YAC. Three ΔHS1 β-YAC transgenic lines were established. ε-globin gene expression was markedly reduced, approximately 16 fold, during primitive erythropoiesis compared to wild-type β-YAC mice, but γ-globin expression levels were unaffected. However, during the fetal stage of definitive erythropoiesis γ-globin gene expression was decreased approximately 4 fold at day 12 and approximately 5 fold at day 14. Temporal developmental expression profiles of the β-like globin genes were unaffected by deletion of 5'HS1. Decreased expression of the ε- and γ-globin genes is the first phenotype ascribed to a 5'HS1 mutation in the human β-globin locus, suggesting that this HS does indeed have a role in LCR function beyond simply a combined synergism with the other LCR HSs.

Keywords

LCR; hypersensitive sites; globin; transcription; erythropoiesis

INTRODUCTION

Five functional human β-like globin genes are arranged in an order (5'-ε-Gγ-Aγ-δ-β-3') that parallels their sequence of expression during development. Gene proximal regulatory elements are sufficient to direct the correct developmental expression of the globin genes during development [1;2;3;4], but a major component of the β-globin locus, required for high level gene expression, is the locus control region (LCR), located 6 to 22 kb upstream of the ε-globin gene. The LCR is composed of four erythroid cell-specific DNase I hypersensitive sites (5'HS1-HS4) and one ubiquitous HS (5'HS5) [5;6;7;8]. Studies in transgenic mice and transient and stable transfection assays demonstrated that the LCR establishes and maintains

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an active β-globin locus chromatin domain, restricts globin gene expression to erythroid cell lineages, enhances globin gene expression and protects the globin genes from negative position effects at the site of transgene integration [7;8;9;10;11;12;13].

The mechanism by which the LCR contributes to stage-specific globin gene switching remains unclear. Recent evidence suggests that the LCR functions as a holocomplex, in which the HSs and their bound protein complement function additively and synergistically, within an active chromatin hub (ACH) structure formed between the LCR, globin genes and distant 5' and 3' HSs [14;15;16;17;18]. Other experiments in transgenic mice clearly demonstrate that the 5'HS2, 5'HS3 and 5'HS4 sites retain distinct and individual functions within this higher order complex [19;20;21;22;23;24;25;26]. The 200-400 bp core regions of each of these sites are capable of conferring position-independent expression upon linked constructs in transgenic mice [11;27;28;29;30]. In addition, each of the four HSs has a functionally distinct role during development, with the possible exception of 5'HS1, which appears to play a minor role. 5'HS2 functions as a major enhancer throughout development; 5'HS3 is necessary for ε-globin gene expression in the embryonic yolk sac and γ-globin gene expression in the fetal liver; and 5'HS4 is required for proper β-globin gene expression in adults [30]. The fact that the distinct role these HSs perform is in the context of a LCR holocomplex, within the higher order ACH structure, is supported by globin gene activation studies of primary transcripts using *in situ* hybridization that showed only one promoter is active at any given time [14;22]. These data suggested a model in which the LCR holocomplex, within the ACH, "flip-flops" between the globin gene promoters by looping out the intervening DNA [14].

Transgenic mice harboring β-globin locus yeast artificial chromosomes (β-YACs) have been used to discern the function of individual HSs and to understand how the LCR interacts with individual globin genes during development [15;19;23;24;31;32;33]. These mice show correct regulation of the human globin transgenes, presumably because all of the human *cis*-regulatory elements are present and recognized by the murine *trans*-acting environment. In β-YAC transgenic mice, the ε-globin gene is regulated like its murine orthologue ε^{y}-globin; expression occurs during primitive erythropoiesis in the yolk sac. The human γ-globin genes are regulated like their murine orthologue βh1-globin; expression is observed during primitive erythropoiesis in the yolk sac. However, unlike βh1-globin, γ-globin does not switch off with the onset of definitive erythropoiesis in the fetal liver; the genes continue to be expressed during this stage. Human β-globin gene expression occurs only during definitive erythropoiesis similar to its murine orthologues β^{min}- and β^{maj}-globin. We previously reported results obtained from transgenic mice carrying a 213 Kb β-YAC harboring large deletions of 5'HS3 and 5'HS2 cross-species conserved sequences, the core elements of 5'HS4 and 5'HS3, and 5'HS3 GT box point mutations [18;23;24;32;34]. In general, our results indicated that LCR interaction with the downstream β-like globin genes is developmental stage specific. In addition, deletion of the HS cores had a more catastrophic effect on globin gene expression than their larger HS deletion counterparts, supporting the hypothesis that the LCR functions as a holocomplex or ACH, in which the core elements form an active site that is disrupted by core deletions or point mutations, but not by larger deletions of the HSs, which allow the LCR to adopt an alternate, albeit less functional, holocomplex [14;15].

In this work, we continue our analysis of HS function by engineering a large deletion of 5'HS4 to complement our published 5'HS4 core deletion and complete testing of the hypothesis that the LCR 5'HS cores form an active site. We predict, and demonstrate in this study, that the larger deletion results in a weaker phenotype than the corresponding 5'HS4 core deletion. In addition, we test the effect of a 5'HS1 deletion on globin gene expression and show a globin gene expression phenotype associated with this mutation. The contributions of 5'HS4 and 5'HS1 to overall LCR function were assessed by deleting a 2.9 Kb or a 0.8 Kb fragment, respectively, encompassing regions of evolutionarily-conserved sequence similarity, from a

213 kb β-YAC. Globin gene expression was analyzed during development in F_2 progeny of transgenic mice.

MATERIALS

YAC constructs

A 213 Kb β-YAC containing the human β-globin locus (Figure 1), was utilized for all experiments [35;36]. This β-YAC was previously estimated to be 248 Kb, but with a nearly complete sequence of the human β-globin locus region now available (http://globin.cse.psu.edu) [37], the size was more accurately determined to be 213 Kb. An approximately 6 Kb sequence gap still exists downstream of 3'HS1 in latest release of the human genome sequence. The numerical coordinates used in construct descriptions are based upon the GenBank file U01317 unless indicated otherwise. All of the YAC modifications were introduced by the yeast integrating plasmid (YIP)-mediated "pop-in", "pop-out" or two-step gene replacement methods of homologous recombination as previously described [38].

Δ5′HS4 β-YAC

Plasmid pHSIV (gift from Dr. Q. Li) containing the β-globin LCR 5'HS4 region (GenBank coordinates 308-3717) was digested with *Stu*I to remove a 2882 basepair (bp) fragment (GenBank coordinates 556-3438) and re-ligated to produce pHSIV Δ*Stu*I (556-3438). An 1166 bp *Hind*III fragment containing the yeast uracil gene URA3 was isolated from YEP24-neo (gift from Dr. C. Huxley), the ends were made blunt by filling with *E. coli* polymerase I Klenow fragment (Roche, Indianapolis, IN) and it was ligated into *Stu*I-cut and calf intestinal alkaline phosphatase-treated (CIAP, Roche, Indianapolis, IN) pHSIV Δ*Stu*I (556-3438) to produce pHSIV Δ*Stu*I (556-3438)::URA3. A 1700 bp *Bam*HI fragment containing the 5'HS4 basepairs 308-556 and basepairs 3438-3717 with the URA3 cassette in between these regions was isolated from this plasmid and transformed into yeast; selection was for uracil prototrophy. For the second transformation and recombination, a 534 bp *Bam*HI fragment containing the same portions of 5'HS4 was purified from digested pHSIV Δ*Stu*I (556-3438); selection was for 5-FOA resistance.

Δ5'HS1 β-YAC

A 6532 bp *Eco*RI 5'HS1 fragment (GenBank coordinates 10,947-17,479) isolated from pHSI (gift from Dr. Q. Li) was ligated into pUC19, in which the *Hind*III site previously was ablated by restriction enzyme digestion, Klenow enzyme filling and religation. The resultant plasmid was digested with *Nco*I (GenBank coordinate 12,942) and *Hind*III (GenBank coordinate 13,769) to remove 827 bp of 5'HS1, the ends were made blunt with Klenow enzyme, and the episome was re-ligated. The truncated 5705 bp *Eco*RI 5'HS1 fragment was purified and ligated into the *Eco*RI-digested and CIAP-treated YIP vector pRS406 (Stratagene, La Jolla, CA) to produce pRS406 ΔHS1. This YIP was linearized with *Hpa*I (GenBank coordinate 11,978) prior to transformation of β-YAC-bearing yeast.

YAC purification and transgenesis

Purification of β-YAC DNAs for microinjection was performed as described previously [39]. Briefly, pulsed-field gel electrophoresis (PFGE) of preparative high-mass DNA agarose plugs was used to fractionate yeast chromosomes and the β-YAC, followed by electrophoretic concentration of the YAC DNA in a 4% low-melting point agarose gel, β-agarase digestion of the concentrated YAC DNA plugs, and filtration of the liquefied agarose solution through a 0.22 μm syringe filter in high-salt microinjection buffer. Purified and filtered YAC DNAs were microinjected into fertilized mouse oocytes (C57Bl/6) and then transferred to pseudopregnant foster mothers to produce transgenic mice. Transgenic animals were identified by slot blot

hybridization or PCR analysis of tail-biopsy DNA. Founders were bred with non-transgenic mice to produce F_1 progeny; these in turn were bred to obtain F_2 staged embryos, fetuses and adults. All structure-function analyses were carried out beginning with the F_2 generation.

Structural analysis of transgene integrity and copy number determination

Structural analysis of β-YAC transgenes was performed to assess the integrity and colinearity of transgenes by long-range restriction enzyme mapping (LRRM) as described previously [38]. High molecular weight DNA embedded in agarose was prepared and slices were digested with *Sfi*I. Digested DNA was fractionated by PFGE and transferred to Zeta-probe charged nylon membrane (Bio-Rad, Hercules, CA) by capillary blotting. Strips representing individual lanes of the gel were cut from the blot and each one was hybridized with one of following ^{32}P-radiolabeled probes: 0.7 Kb *Pst*I 5'HS3, 1.9 Kb *Hind*III 5'HS2, 1.8 Kb *Xba*I 5'HS1, 3.7 Kb *Eco*RI ε-globin gene, 2.4 Kb *Eco*RI fragment 3' $^{A}\gamma$-globin gene, 1.0 Kb *Eco*RV Ψβ region, 2.1 Kb *Pst*I fragment 5' δ-globin gene, 0.9 Kb *Eco*RI-*Bam*HI fragment 3' β-globin gene, 1.4 Kb *Xba*I DF10 (3'HS1), 1.9 Kb *Bgl*II HPFH3, 0.5 Kb *Hind*III H500, and 1.5 Kb *Eco*RI-*Bgl*II HPFH6. DNA probes were labeled using the Decaprime II kit (Ambion, Austin, TX) following the manufacturer's instructions.

The presence of 5'HS5 was confirmed by Southern blot hybridization using a ^{32}P-radiolabeled 3.2 Kb *Bam*HI fragment (Globin Gene Server human β-globin locus coordinates 215,119-218,342) as a probe [37]. Digestion with *Sfi*I (218,064-226,104) produces a diagnostic 8.0 Kb fragment that contains 5'HS5 and part of 5'HS4; this fragment is immediately adjacent to the 115 Kb *Sfi*I fragment (226,064-341,399) bearing the remainder of the β-globin locus beginning within 5'HS4.

Transgene copy numbers were determined by Southern blot hybridization using LCR 5'HS3, γ-, and β-globin gene fragment probes co-radiolabeled with a mouse Thy 1.1 gene fragment as described previously [32]. Probe specific activity was corrected and comparison of hybridization signal intensity between human globin transgenes and the endogenous diploid murine Thy1.1 gene gave an estimate of transgene copy number. Radioactive signals were quantitated using a phosphorimager (Packard Instruments, Meriden, CT).

RNase protection analysis (RPA)

Total RNA was isolated from yolk sac, fetal liver and blood of developmentally-staged transgenic F_2, or later, embryos, fetuses and adults by the method of Chomczynski and Sacchi [40] using the RNAgents Total RNA Isolation reagents (Promega, Madison, WI) as previously described [32;41]. Human and murine globin mRNAs were detected by RNase protections performed as described previously [41;42]. Antisense RNA probes were prepared using the MaxiScript II kit (Ambion, Austin, TX). Template DNAs used to prepare riboprobes to measure endogenous mouse α- and ξ-globins were pT7Moα and pT7Moξ, respectively [13] and to measure human ε-, γ-, and β-globins the templates were pT7Huε( ), pT7$^{A}\gamma^{m}$(170), and pT7β^{m}, respectively [43;44]. Individual human β-like globin gene expression levels were quantitated as a percentage of total human β-like globin gene expression or as a percentage of murine α- and ξ-globin gene expression corrected for transgene and endogenous murine gene copy number using data collected on a Cyclone Phosphorimager and OptiQuant analysis software (Packard Instruments, Meriden, CT). Data include the means and standard deviations from two or more experiments with at least two measurements for each developmental time point. For statistical analysis, the Student *t* test of comparison of means was performed, and differences were considered significant at *P* values of < 0.05.

RESULTS

Δ5'HS4 β-YAC transgenic mice

A 2.9 Kb deletion of human β-globin LCR 5'HS4, which included the 200 bp DNase I HS core [32] and evolutionary conserved flanking sequences [45], was introduced into a 213 KB β-YAC (Figure 1) by the two-step gene replacement method of homologous recombination in yeast as described in Materials. Correct targeting of this sequence alteration to the YAC in the yeast host was confirmed by PCR and standard Southern blot hybridization analysis or LRRM as previously described (data not shown). This mutant β-YAC was purified and microinjected into fertilized C57Bl/6 oocytes to produce transgenic mice using established methodology [36;46]. Eleven transgenics were identified among the 77 founders obtained (14% transgenesis). Of these, five contained intact β-globin loci; three were bred to establish lines.

LRRM analysis assesses the integrity of the human β-globin locus on a 115 Kb *Sfi*I restriction enzyme fragment contained within the 213 Kb β-YAC by hybridizing multiple PFGE lanes with separate probes spanning the β-globin locus. One *Sfi*I site is located near 5'HS4 of the LCR, the other is downstream of 3'HS1 (Figure 1). *Sfi*I fragments smaller or larger than 115 Kb indicate the loss of at least one of the *Sfi*I sites flanking the β-globin genes and the size of the resultant fragment is dependent upon the juxtaposition of the nearest murine genomic *Sfi*I site. PFGE structures of the DNA isolated from β-YAC transgenic mouse lines have shown that these mice usually contain multiple *Sfi*I fragments of different sizes [38;47]. The three Δ5'HS4 β-YAC lines contained two intact copies of the β-globin locus from within LCR 5'HS4 through the HPFH6 breakpoint, well downstream to the 3' β-globin gene enhancer (Figure 2). Additional copies with deletions at the 5' or 3' ends, or both, were observed for all three lines. In all instances, these copies lacked the entire LCR or all the globin genes; thus they did not contribute significantly to total globin transgene expression. In addition, we demonstrated the presence of 5'HS5 and the Δ5'HS4 deletion in all intact locus copies associated with these lines, except for line 38, which lacks 5'HS5, but contains all of 5'HS4 minus the deletion (Figures 3 and 4).

F_2 or later generation transgenics were bred to produce staged conceptuses for analysis of globin gene expression during development. Total RNA was isolated from 10 day yolk sac, 12 and 14 day fetal liver and adult blood for analysis by RNase protection assay (RPA). As shown in Figures 5 and 6 the only significant alteration in gene expression in the mutant Δ5'HS4 β-YAC mice was a slight, but statistically significant decrease of β-globin during early (day 12) fetal liver definitive erythropoiesis ($P < 0.05$; Table 1). This gene expression phenotype somewhat parallels the phenotype observed in Δ5'HS4 core β-YAC transgenic mice [32], but is not as severe as that measured in the core deletion mice. Although not statistically significant, γ-globin exhibits a trend towards increased levels in the fetal liver (Table 1). The mean standard deviation in the Δ5'HS4 β-YAC lines for γ-globin and β-globin during definitive erythropoiesis suggests that expression was not variegated. This deletion had no effect on ε- or γ-globin gene expression during primitive yolk sac erythropoiesis. Completion of the γ-to β-globin gene expression switch was delayed approximately two days, even though the onset of switching was normal (Figure 7).

Δ5'HS1 β-YAC transgenic mice

A 0.8 Kb LCR 5'HS1 deletion was introduced into a 213 Kb β-YAC by YIP-mediated homologous recombination in yeast as described in Materials. Confirmation of correct targeting in yeast and microinjection of purified YAC DNA to produce transgenic mice were performed as described above for the Δ5'HS4 β-YAC construct. Of 113 total founders, nine β-YAC transgenics were obtained (8% transgenesis); two of these carried intact transgenes

and a third contained sequences extending from the LCR through the upstream δ-globin region. These three founders were bred to establish lines.

Structural analyses were performed as described for the Δ5'HS4 β-YAC lines. Line Δ5'HS1-50 contained two complete copies of the β-globin locus that spanned from within 5'HS4 through the H500 breakpoint downstream to the 3' β-globin gene enhancer (Figure 8, panel A). Line Δ5'HS1-92 carried 5 such copies, ending even more 3' at the HPFH6 breakpoint. Line Δ5'HS1-62 did not bear any complete copies, but had 2 copies extending from within 5'HS4 to just 5' of the δ-globin gene and one copy extending from the same 5' end through the ε-globin gene. However, based on our previous experience, ε- and γ-globin expression can be measured as long as an intact LCR is present, even when the β-globin gene is missing. This line also carried 2 additional deleted β-YAC copies that did not contribute to measurable expression; one spanned the ε-globin gene through the HPFH3 breakpoint and the other extended only between the HPFH3 and H500 breakpoints. Additional copies with very minimal locus sequence were also observed in all three lines. We also demonstrated the presence of the 0.8 Kb Δ5'HS1 deletion by two criteria in all intact copies of these three lines (Figure 8, panel B).

Δ5'HS1 β-YAC transgenic mice of the F_2 or later generations were mated to acquire developmentally-staged conceptuses for measurement of human β-like globin synthesis. RNA was isolated and analyzed as described above for Δ5'HS4 β-YAC transgenic mice. As shown in Figure 9 for the hematopoietic tissues and in Figure 10 for blood, only ε-globin gene expression was overtly affected in a negative manner. The temporal pattern of switching, as reflected by β-like globin gene expression in the blood, was largely unaffected by deletion of 5'HS1 (Figure 11), but reduction of ε-globin gene expression is clearly documented during primitive erythropoiesis. In addition, a delay of approximately one day in the γ- to β-globin switch was observed. Table 2 shows a summary of the quantitative data acquired for globin gene expression in erythropoietic tissues during development as exemplified by the sample analysis shown in Figure 9. ε-globin gene expression was decreased approximately 16 fold during yolk sac erythropoiesis compared to wild-type β-YAC mice ($P < 0.05$); γ-globin expression levels were unaffected. However, during fetal definitive erythropoiesis, γ-globin gene expression was decreased approximately 4 fold at day 12 and approximately 5 fold at day 14 ($P < 0.05$). ε-globin mRNA was nearly undetectable at day 8 in either the yolk sac or RBCs (Figures 9 and 10, respectively). This is the first report of a phenotype associated with deletion of LCR 5'HS1 and it is reminiscent of that associated with our 5'HS3 core deletion [24].

DISCUSSION

Previous work demonstrated that 5'HS4 enhanced β-globin gene expression in a position-independent, copy number-dependent manner in transgenic mice, but not in stably transfected MEL cells [11;28]. Using a recombinant construct linking 5'HS4 to a cosmid containing the $^{G}\gamma$ through the β-globin genes in transgenic mice, Fraser et al. [30] showed that the γ-globin genes were poorly expressed during embryonic and fetal erythropoiesis, but that β-globin was efficiently expressed during fetal and adult erythropoiesis, suggesting that the preferred stage of activation by this 5'HS was in the adult. In addition, 5'HS4 by itself had no effect on expression of a stably- or transiently-transfected ε-globin gene reporter in K562 cells [48], indicating that this HS has no enhancer function on its own, but may play a role in the chromatin-opening function of the LCR during development. No alteration in gene expression was obtained in transgenic mice containing a 72 Kb whole locus construct with an 875 bp deletion of 5'HS4 [22]. Studies in 150 Kb β-YAC transgenic mice carrying a 5'HS4 core deletion showed catastrophic disruption of globin gene expression at all erythroid developmental stages, despite the presence of all the other 5'HSs in the YAC transgenes, a

phenotype much more severe compared with previous data. [19]. However, the ectopically located transgenes may have been sensitive to position effects [49].

Previous results from our 213 Kb β-YAC transgenics showed that a 280 bp deletion of the 5'HS4 core resulted in a significant decrease of γ- and β-globin gene expression during definitive erythropoiesis in the fetal liver and a decrease of β-globin gene expression in adult blood [32]. No effect on globin gene expression was observed during embryonic erythropoiesis. Variable (position-dependent) β-globin gene expression was observed among the transgenic lines during definitive erythropoiesis. From these data, we concluded that the 5'HS4 core element was required for globin gene expression only during definitive erythropoiesis and that it functioned as a chromatin-opening element and/or as a γ-/β-globin enhancer in definitive erythroid cells. In contrast, this study showed only a moderate phenotype associated with a larger, 2.9 Kb deletion of 5'HS4. It caused a slight decrease in β-globin gene expression during the early fetal stage of definitive erythropoiesis: the temporal pattern of globin gene expression was largely unaffected; and position effect variegation (PEV) was not observed.

The 5'HS4 data are consistent with other studies demonstrating that HS core deletions are more deleterious to globin gene expression than larger HS deletions that include both core and flanking sequences. A targeted 2.7 Kb deletion of 5'HS4 in the murine genome led to reduced transcription (up to 27%) of predominantly the adult globin genes in mice [25]. Embryonic gene expression was not significantly affected and the pattern of globin gene expression was not altered. This modest phenotype is consistent with that of the Δ5'HS4 β-YAC mice in this study. It is possible that a smaller, core-only 5'HS4 deletion would have a more profound effect on mouse globin gene expression. Taken together these studies support the existence of an LCR holocomplex structure, as part of an active chromatin hub, in which the 5'HS cores form an active site and the flanking regions dictate the holocomplex conformation. Until recently, all of the 5'HS deletions introduced into the murine β-globin locus were large (2.3 Kb 5'HS1, 1.1 Kb 5'HS2, 2.3 Kb 5'HS3, 2.7 Kb 5'HS4, 3.5 Kb 5'HS5-6) and although the effect on globin gene expression profiles was negative, it was modest in all cases [20;21;25;50]. The phenotypic similarity to the large deletions of 5'HSs within the human locus lends support to the holocomplex model. However, Hu et al. [26] recently produced a targeted deletion of the 328 bp 5'HS2 core in the murine β-globin locus. These mutant mouse lines exhibited an expression pattern nearly identical to the 1.1 Kb 5'HS2 deletion mice. In contrast, a 375 bp human 5'HS2 core deletion [15] in β-YAC transgenic mice was far more catastrophic on globin gene expression than the larger 1.9 Kb deletion [23]. Thus, the holocomplex model may not apply to the mouse β-LCR.

Most interesting was the effect of a 0.8 Kb 5'HS1 deletion, in which ε-globin gene expression was significantly reduced during primitive erythropoiesis and γ-globin was decreased during fetal definitive erythropoiesis. These are the first major phenotypes ascribed to a human 5'HS1 mutation, suggesting that this HS does indeed have a more important role in LCR function than previously thought. This phenotype mirrors that observed in β-YAC transgenic mice containing a deletion of the 5'HS3 core [24], although the 5'HS3 core mutation had the additional phenotype of variegated β-globin gene expression during adult definitive erythropoiesis. The concordance of phenotypes between these two mutations suggests that trans-acting factors bound to 5'HS3 and 5'HS1 cooperatively participate in activation of these two genes at the developmental stages in which they are negatively affected by mutation. Alternately, the 800 bp 5'HS1 deletion may mimic the 200-300 bp 5'HS2, 3 or 4 core deletions and catastrophically disrupt the LCR holocomplex resulting in a gene expression phenotype that closely resembles the 5'HS3 core deletion [18]. We cannot exclude the possibility that the LCR lacking 5'HS1 may be susceptible to the chromatin structure surrounding integration sites.

A patient was described with a γδβ-thalassemia resulting from what is now referred to as the Hispanic deletion, in which LCR 5'HSs 2-5 and a substantial amount of upstream sequence (~ 30 Kb total) were removed, but 5'HS1was intact [51]. Although the phenotype of this patient confirmed the significance of the LCR HSs in regulating globin gene expression, it also demonstrated that on its own 5'HS1 was not sufficient to mediate LCR function. Subsequently, a family was identified with a 3 Kb deletion (Italian deletion) of sequences upstream of the ε-globin gene that included 5'HS1 and co-segregating β^0-thalassemia [52]. The deletion was linked in *cis* to a structurally and functionally normal β-globin locus, suggesting that this HS was not necessary for β-globin gene activity *in vivo*. These data suggest that 5'HS1 may be dispensable for LCR function. However, the readout in these patients was fetal HbF or adult HbA. In the case of the Italian deletion, an HPFH deletion was linked to the β-thalassemia mutation and would have masked the effect of the 5'HS1 mutation on γ-globin gene expression. Phenotypes associated with ε- or γ-globin would not have been observed. In addition, conservation of 5'HS1 sequences between species suggests that this *cis*-regulatory element must play an important role in globin locus structure in some capacity during hematopoiesis [45].

In early studies, DNAse I-hypersensitivity of 5'HS1 was observed in erythroid cells (formerly called HS -6.1, location relative to the ε-globin gene); however, in non-erythroid cells, this site was not formed, but two alternate ones were detected at -7.2 and -5.2 in these lineages [53]. The -7.2 and -5.2 HSs did not influence globin gene expression. Ultimately, the final lineage reflected which set of sites remained sensitive, suggesting that 5'HS1 influenced, or was a consequence of, lineage choice during development. Other, more recent data demonstrated that during differentiation, all LCR hypersensitive sites were observed early in hematopoiesis prior to erythroid lineage formation [54;55] and that activation of the LCR in progenitor cells may be required for normal LCR function [56]. Thus, 5'HS1 may have a role in determining erythroid potential [54]. Supporting this hypothesis are data demonstrating that locus activation in human β-globin locus cosmid transgenic mice bearing a 5'HS1 deletion appears to be a cellular timing phenomenon, reflecting a possible function of 5'HS1 in differentiation stage or cell cycle phase control of heterochromatin-mediated gene silencing [22].

In addition to naturally occurring mutations in humans, data generated from a variety of transgenic mice implied that 5'HS1 played only a minor role in LCR function and globin gene expression. Early experiments indicated that: 1) α-globin was not expressed in a 5'HS1-α-globin transgenic mouse [57]; 2) 5'HS1 contributed to full expression within the context of the LCR, but only low expression was observed when just 5'HS1 was present to drive globin gene expression [58]; and 3) LCR 5'HS1 was not important for adult globin gene expression [11]. By itself, 5'HS1 exerted minimal enhancer function throughout development relative to the other 5'HSs and was weakest in the yolk sac, although an increased copy number-dependent level of linked gene expression was observed [30]. Deletion of 5'HS1 in linked-cosmid whole locus constructs resulted in a loss of copy number dependence and produced substantial sensitivity to position effects in transgenic mice [22]. In summary, the β-LCR 5'HS1 confers position-independent, copy number-dependent low-level gene expression in transgenic mice, is unable to enhance gene expression in transiently or stably transfected cell lines and shows no chromatin domain-opening or insulator activity [45]. Until this work, no stage-preference was observed, but clearly ε-globin gene expression in the yolk sac and γ-globin expression in the fetal liver requires 5'HS1 for normal levels of synthesis. More recent data confirms the requirement for 5'HS1 to obtain full expression of β-globin transgenes directed by the LCR in gene therapy constructs, suggesting that the role of 5'HS1 may be primarily architectural and designed for alignment of regulatory elements within the LCR and the globin gene promoters/enhancers [59]. Targeted deletion of 5'HS1 in the murine β-globin locus substantially reduced expression of linked genes, but did not affect the temporal pattern of globin gene expression, nor did this HS appear to exhibit developmental stage specificity [25]. This study in

combination with other knockout mice studies indicates that the LCR HSs form independently and appear to contribute additively to overall gene expression from the locus. While our data demonstrate some similarity to these findings, the human locus in transgenic mice also shows differences, such as stage-specificity for enhancement of ε-globin, but not γ-globin, during primitive erythropoiesis and enhancement of γ-globin gene expression during fetal definitive erythropoiesis. In addition, the compendium of transgenic studies suggests that the HSs synergize. The additive and synergistic modalities of HS action need not be mutually exclusive, although these observations may reflect very real species differences.

Although the mouse and human β-LCR 5'HSs share significant sequence homology, several differences in their functional mechanisms have arisen. The human 5'HSs appear to have specific functions in mediating LCR function; these can be locus-wide, gene-specific or developmental stage-specific, whereas the mouse 5'HSs appear to lack distinct, separable functions. Deletion of the entire LCR has highly deleterious effects, producing β-thalassemia in humans [51], but a similar deletion in the murine locus is far less catastrophic [60]. Thus, mouse and human LCR deletions produce different effects on chromatin structure and globin gene expression, but the functional mechanisms underlying these differences remain unknown. These different observations may simply reflect the dichotomy between studying human transgenes versus endogenous mouse loci or they may be revealing real functional differences. Studies in human erythroid cell lines in which gene-targeting experiments can be performed become essential to reconciling these data.

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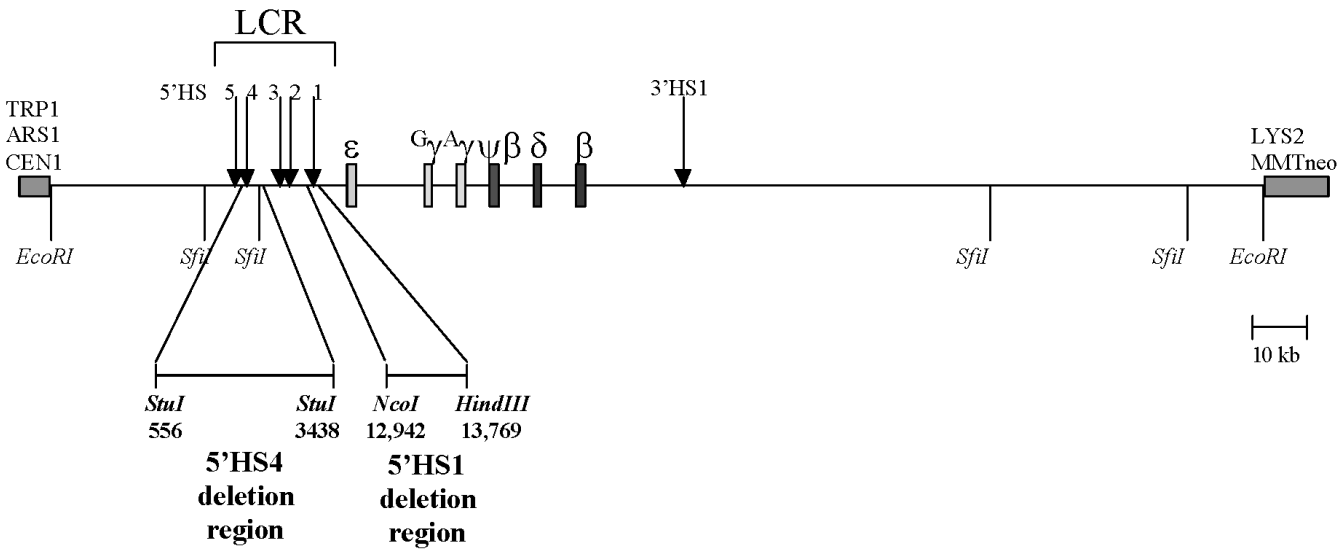

Figure 1. Schematic diagram of Δ5'HS1 and 5'HS4 β-YAC

The human globin locus and flanking genomic sequences are indicated as a line; globin genes are shown as boxes and YAC vector sequences are indicated as boxes at the left and right ends. The LCR, its associated DNAseI-hypersensitive sites (HSs), and the globin genes are shown above the line; yeast chromosomal elements and selectable markers are listed above the boxes. Restriction enzyme sites are illustrated below the line. The deletions are displayed below the YAC diagram and their position within the β-YAC is shown. An 827 bp *Nco*I-*Hind*III fragment (GenBank coordinates 12,942-13,769) was deleted for 5'HS1 and a 2882 bp *Stu*I fragment (GenBank coordinates 556-3438) was deleted for 5'HS4.

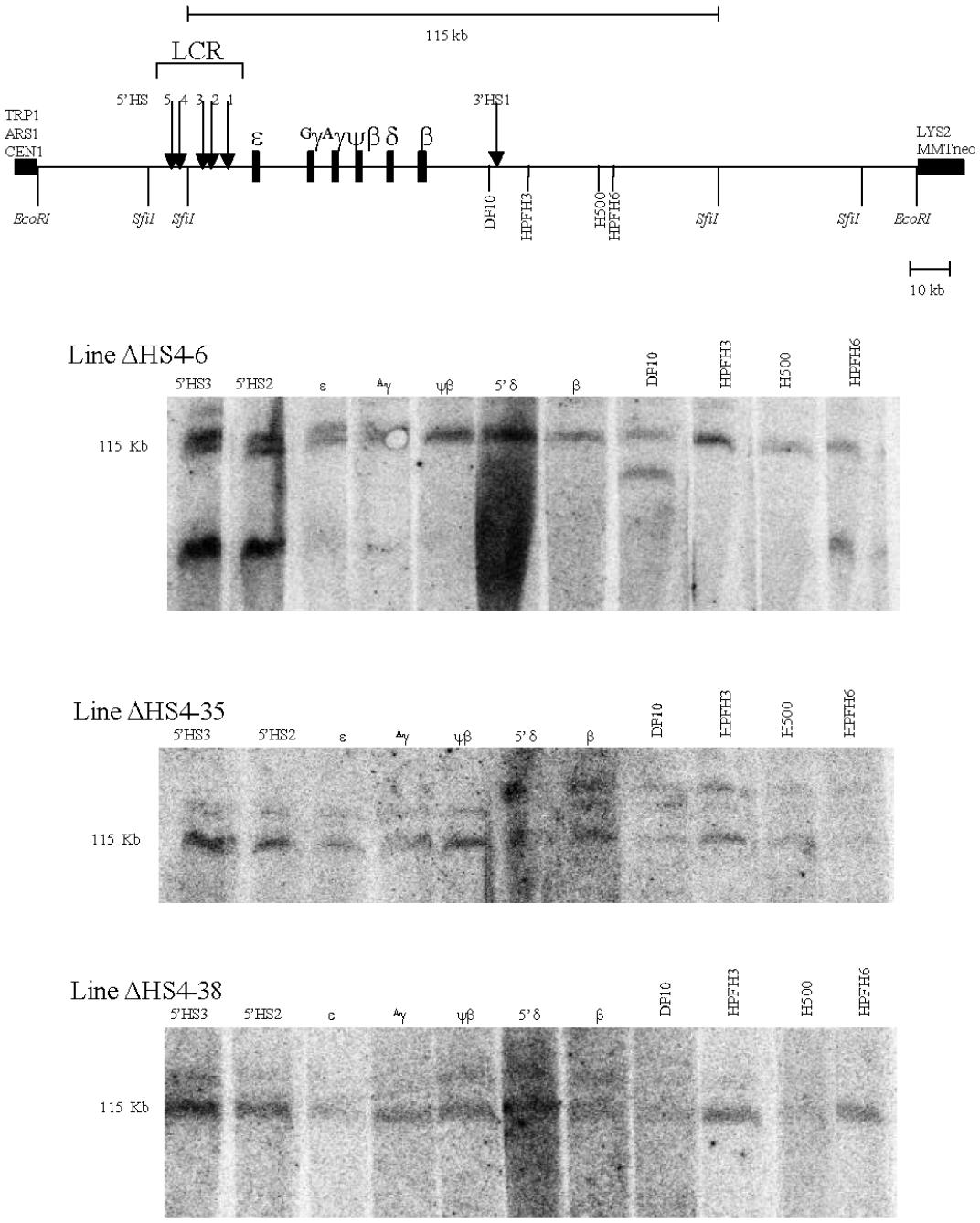

Figure 2. PFGE structural analysis of three Δ5'HS4 β-YAC transgenic mouse lines

Structural analysis was performed as described in Materials. A schematic diagram of the YAC is shown at the top of the figure; above that, the diagnostic 145 Kb *Sfi*I fragment encompassing most of the β-globin locus is illustrated. This *Sfi*I fragment is larger than the normal 140 Kb *Sfi*I fragment because the 5'HS4 deletion removes one of the diagnostic *Sfi*I sites, but juxtaposes another upstream of 5'HS5 (see Figure 1). Autoradiographs of lines Δ6, Δ35 and Δ38 are displayed. The probes used are listed above each autoradiograph. The location of the145 Kb *Sfi*I fragment is indicated on the left. All three lines contain two intact copies of the locus. Copy numbers were validated by standard Southern blot analysis.

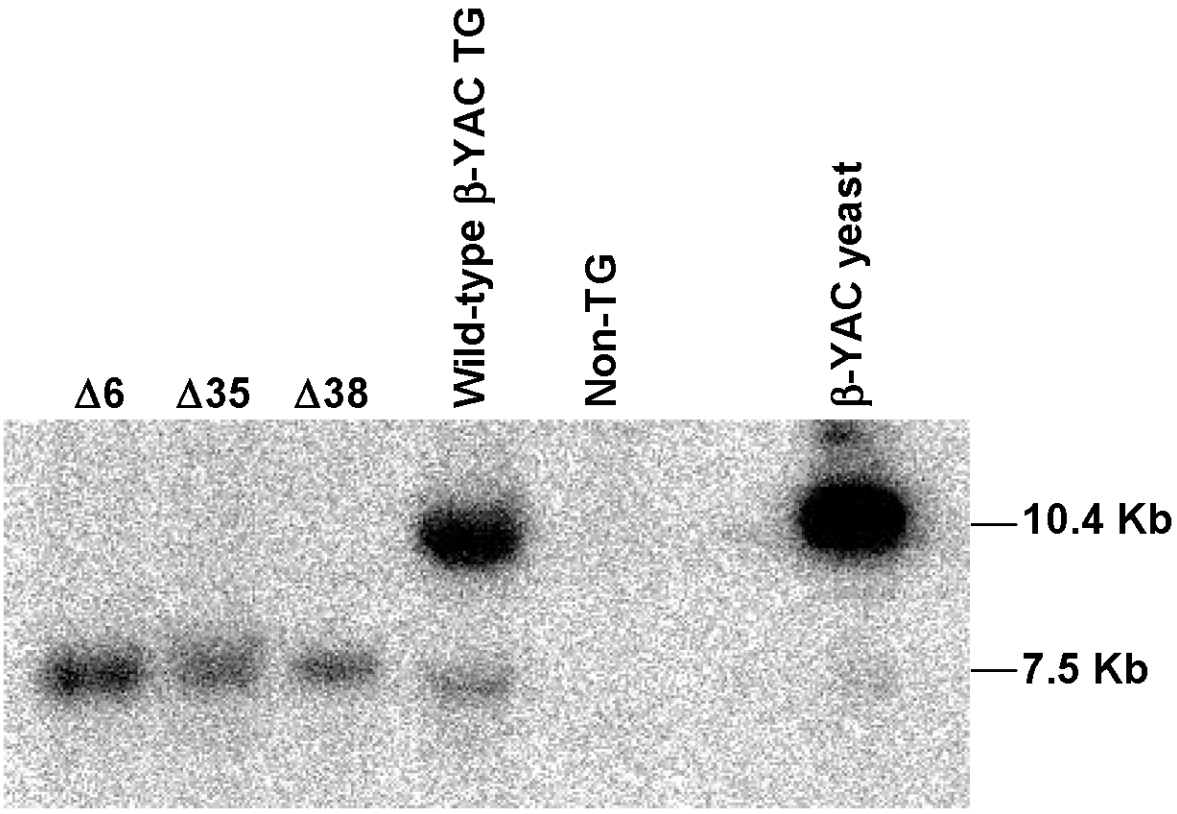

Figure 3. Confirmation of deletion of 2.9 kb *Stu*I fragment in 5'ΔHS4 lines

Southern blot hybridization analysis of *Eco*RI-digested DNA from wild-type and Δ5'HS4 transgenic lines. Wild-type lines produce a 10.4 Kb fragment (*Eco*RI sites at GenBank coordinates 1 and 10,424); mutant lines produce a 7.5 Kb fragment due to deletion of 2.9 Kb (GenBank coordinates 556-3438).

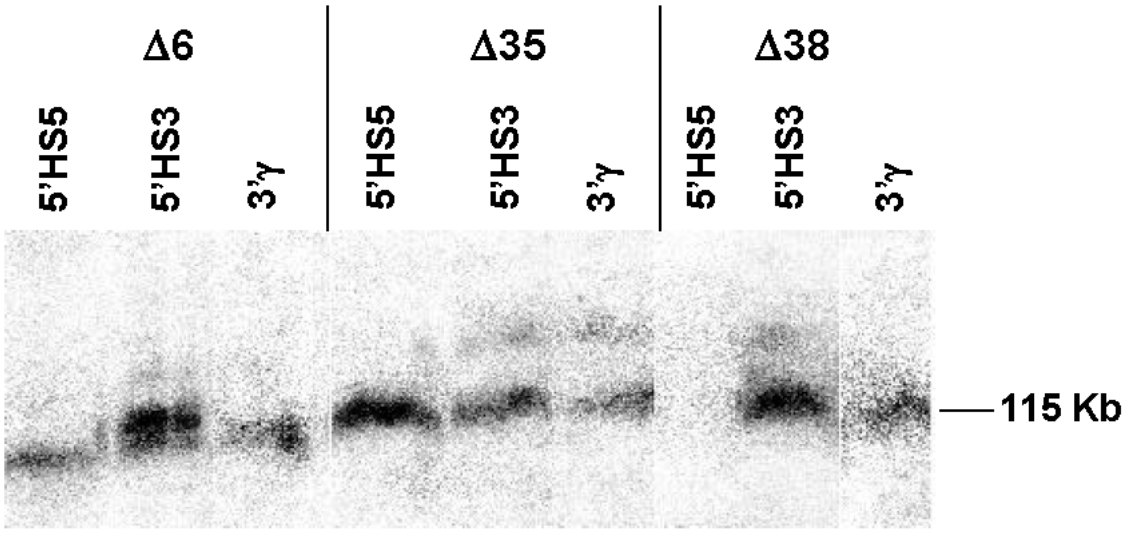

Figure 4. Confirmation of presence of 5'HS5 in Δ5'HS4 lines

Southern blot hybridization analysis of *Sfi*I digested samples fractionated by PFGE (see Figures 3 and 4) demonstrates linkage of 5'HS5 to the remainder of the β-globin locus in lines Δ6 and Δ35. Although in the Δ38 line 5'HS5 is not contiguous with the β-globin loci, at least 556 bp DNA is present upstream of the 5'HS4 deletion as shown by the correct size *Eco*RI fragment displayed in Figure 3.

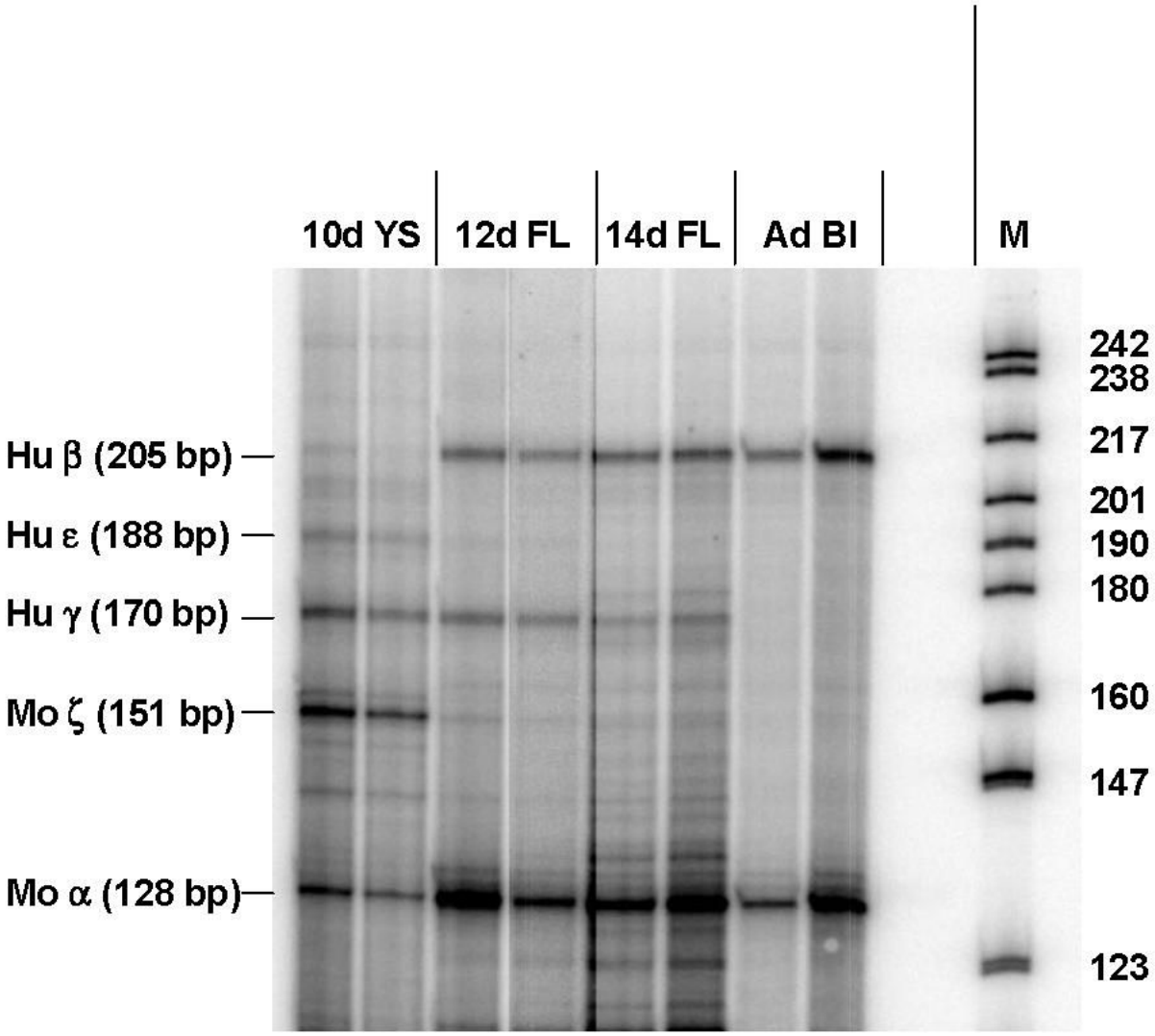

Figure 5. β-like globin gene expression in Δ5'HS4 β-YAC transgenic line 6, 35 and 38 hematopoietic tissues during development

Total RNA was isolated from staged fetuses and subjected to RNAse protection. The tissues and days after conception are shown at the top of the autoradiographs. Anti-sense RNA probes for human ε-, γ- and β- and mouse α-and ζ-globins were utilized. Protected fragments are displayed on the left: Hu β, 205 bp; Hu ε, 188 bp; Hu γ, 170 bp; Mo ζ, 151bp; Mo α, 128 bp. pBR322 *Msp*I DNA size markers are shown on the right. Control lanes are indicated and show the location of the protected fragments. YS, yolk sac; FL, fetal liver; Bl, blood. Subtle quantitative differences were observed between wild-type and mutant β-YAC lines (see Table 1).

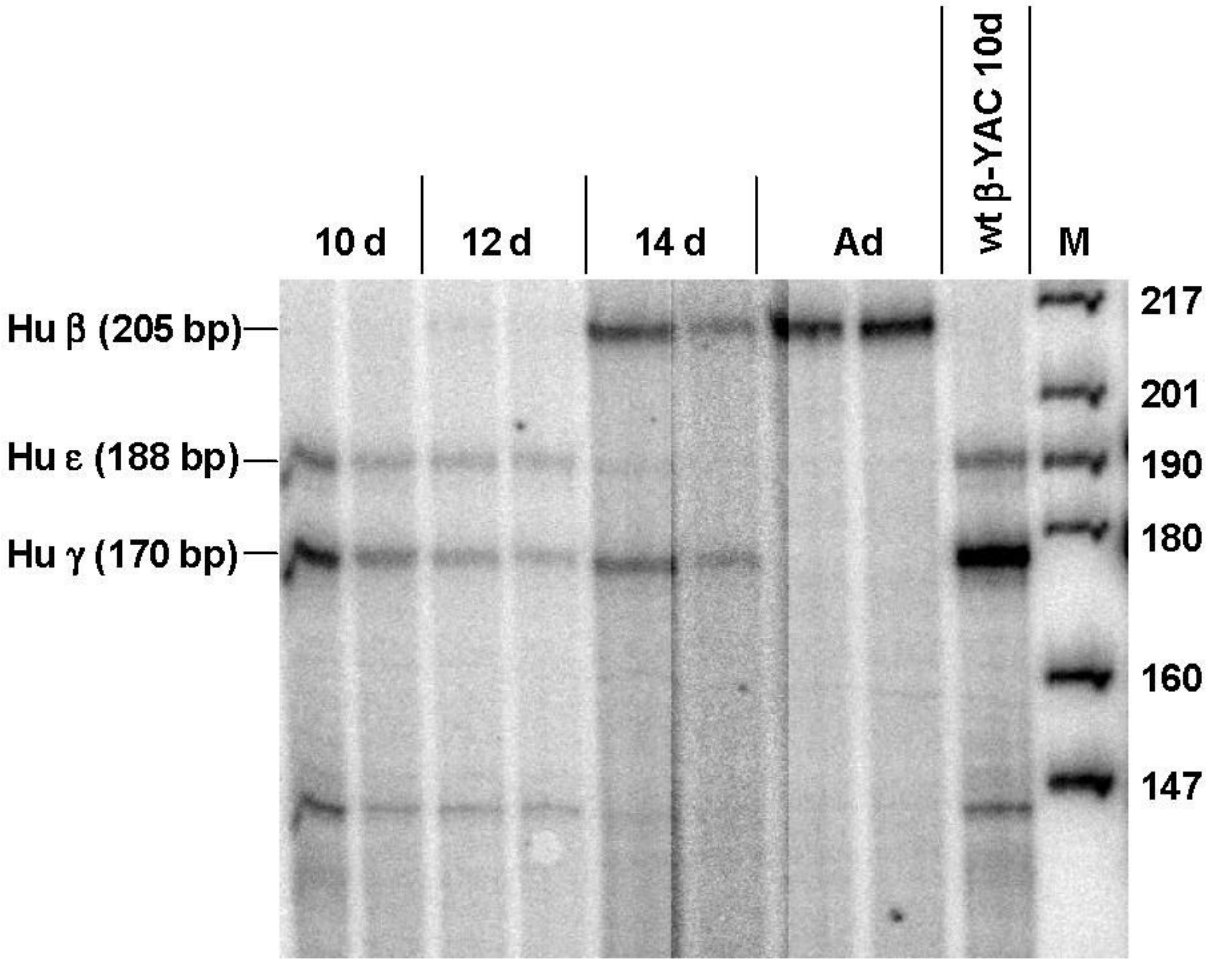

Figure 6. Temporal pattern of globin gene switching in blood of Δ5'HS4 β-YAC transgenic mice
Labeling is as for Figure 5. Qualitative differences between wild-type and mutant β-YAC lines were not observed.

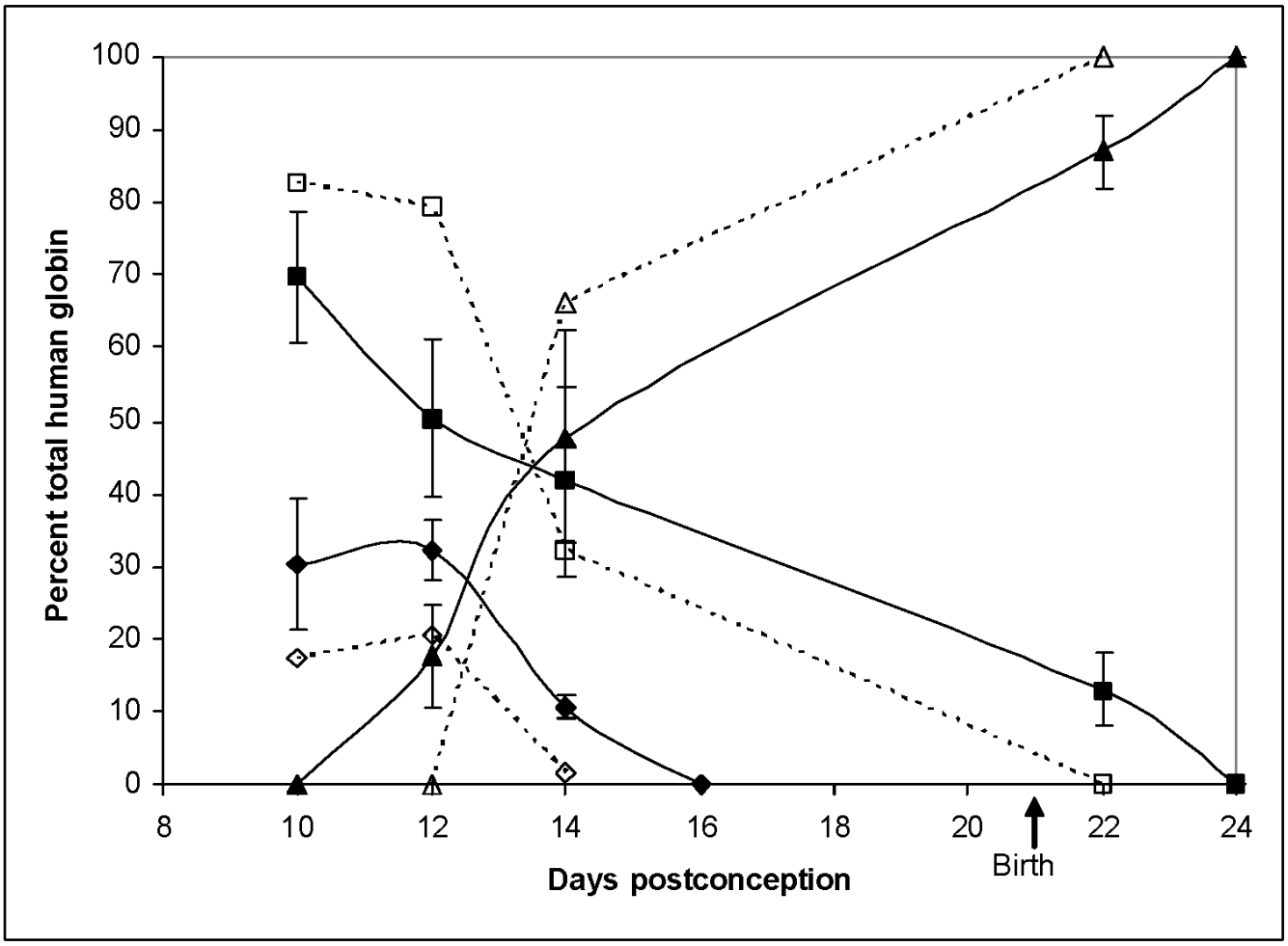

Figure 7. Globin gene switching during development in Δ5'HS4 β-YAC lines

RNA samples prepared from blood of staged fetuses and adults were assayed by RPA as described. On the y-axis, expression of each human globin gene is displayed as a percentage of total globin transcribed. The developmental days post-conception are shown on the x-axis. Experimental values for the Δ5'HS4 lines are the means and standard deviations of all three lines with a minimum of two conceptuses for each line at each time point from 3 independent experiments. Standard deviations are not shown for the wild-type β-YAC data, so that the data in the figure would not be difficult to read; the mean values plotted are derived from 3 independent experiments, with duplicate samples for each time point in each experiment. The mean values plotted are well within norms of our previously published reports using this line. Note the longer switching period observed in the Δ5'HS4 lines. Open symbols, wild-type β-YAC line 26223; closed symbols, Δ5'HS4 β-YAC lines. Diamonds, ε-globin; triangles, γ-globin; squares, β-globin.

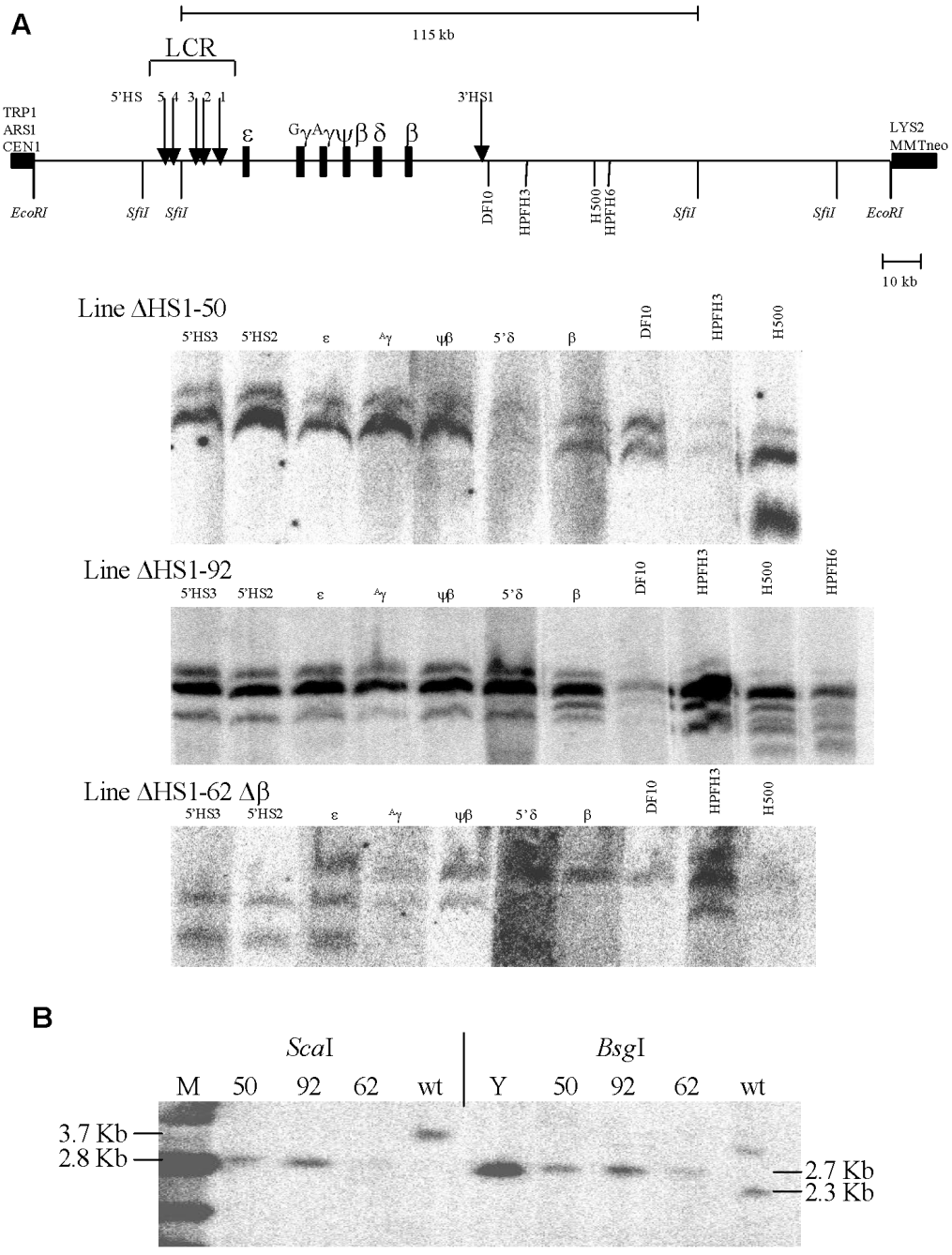

Figure 8. Structural analysis of Δ5'HS1 β-YAC transgenic mouse lines. Panel A: PFGE structural analysis

Labeling is as for Figure 2. Line 50 contains 2 intact loci; Line 92 contains 5 intact loci. Line 62 contains 4 incomplete loci; two extend from the LCR through the 5' upstream region of the δ-globin gene and one extends from the LCR through the ε-globin gene. **Panel B: Confirmation of 5'HS1 deletion (0.8 Kb)**. Southern blot hybridization analysis of *Sca*I- (left) or *Bsg*I-digested (right) DNA from Δ5'HS1 or wild-type β-YAC transgenic lines. For *Sca*I digests, wild-type lines produce a 3.7 Kb fragment (*Sca*I sites at GenBank coordinates 10,404 and 14,086); mutant lines produce a 2.8 Kb fragment due to deletion of 0.8 Kb (GenBank coordinates 12,942-13,769). For *Bsg*I digests, a 2.3 Kb band is observed for wild-type β-YAC

lines (GenBank coordinates 10,597-12,939). Δ5'HS1 β-YAC lines display a 2.7 Kb fragment that results from deletion of the *Bsg*I site at 12,939 and juxtaposition of the next nearest *Bsg*I site located at 14,242 (14,242 - 10,597 = 3645 bp; 3645 bp - 827 bp Δ5'HS1 deletion = 2718 bp)

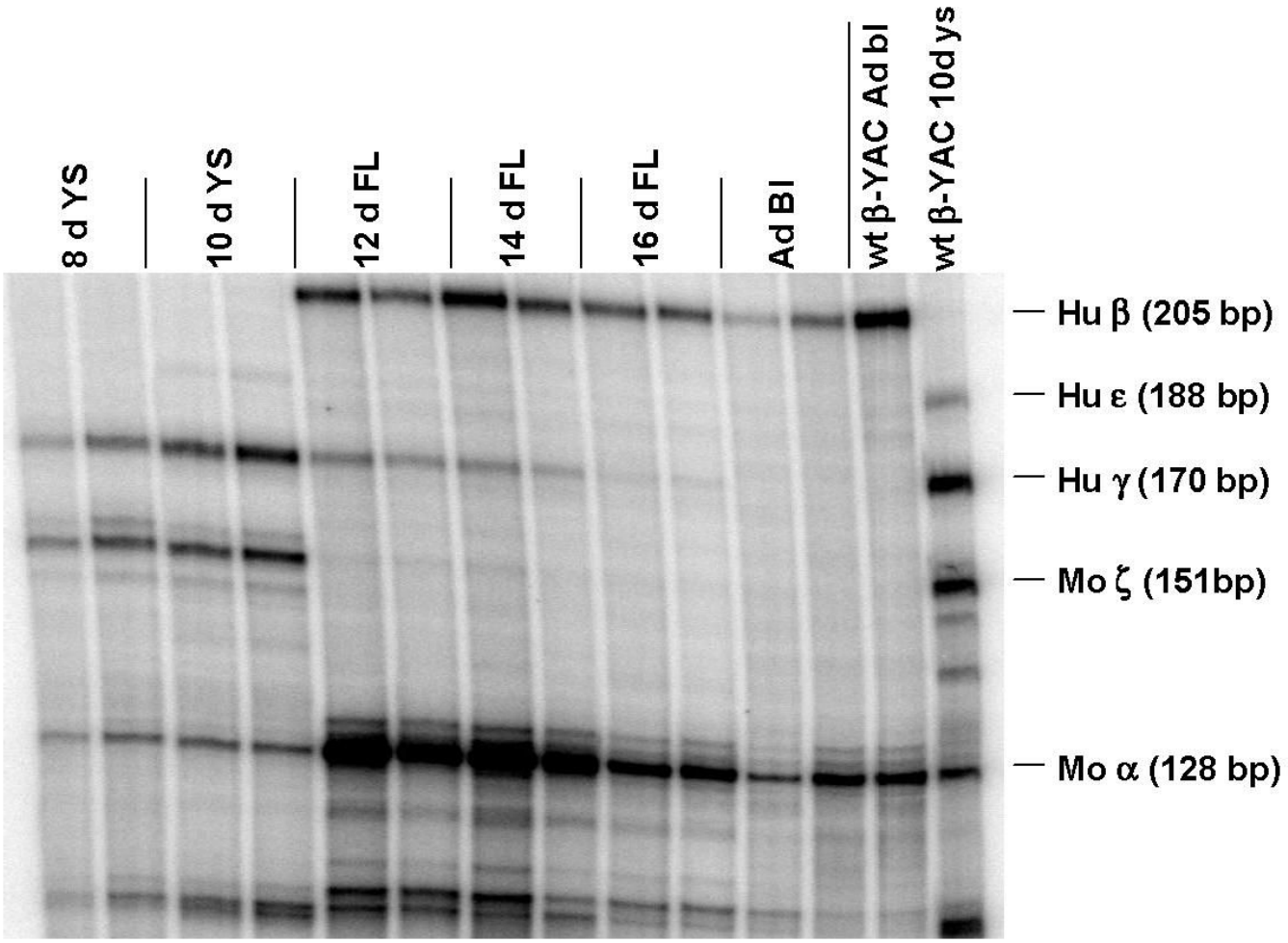

Figure 9. Human globin transgene expression in hematopoietic tissues of Δ5'HS1 β-YAC transgenic lines

Labeling is the same as denoted in Figure 5. Note the decrease of ε-globin gene expression in the Δ5'HS1 lines (see Table 2).

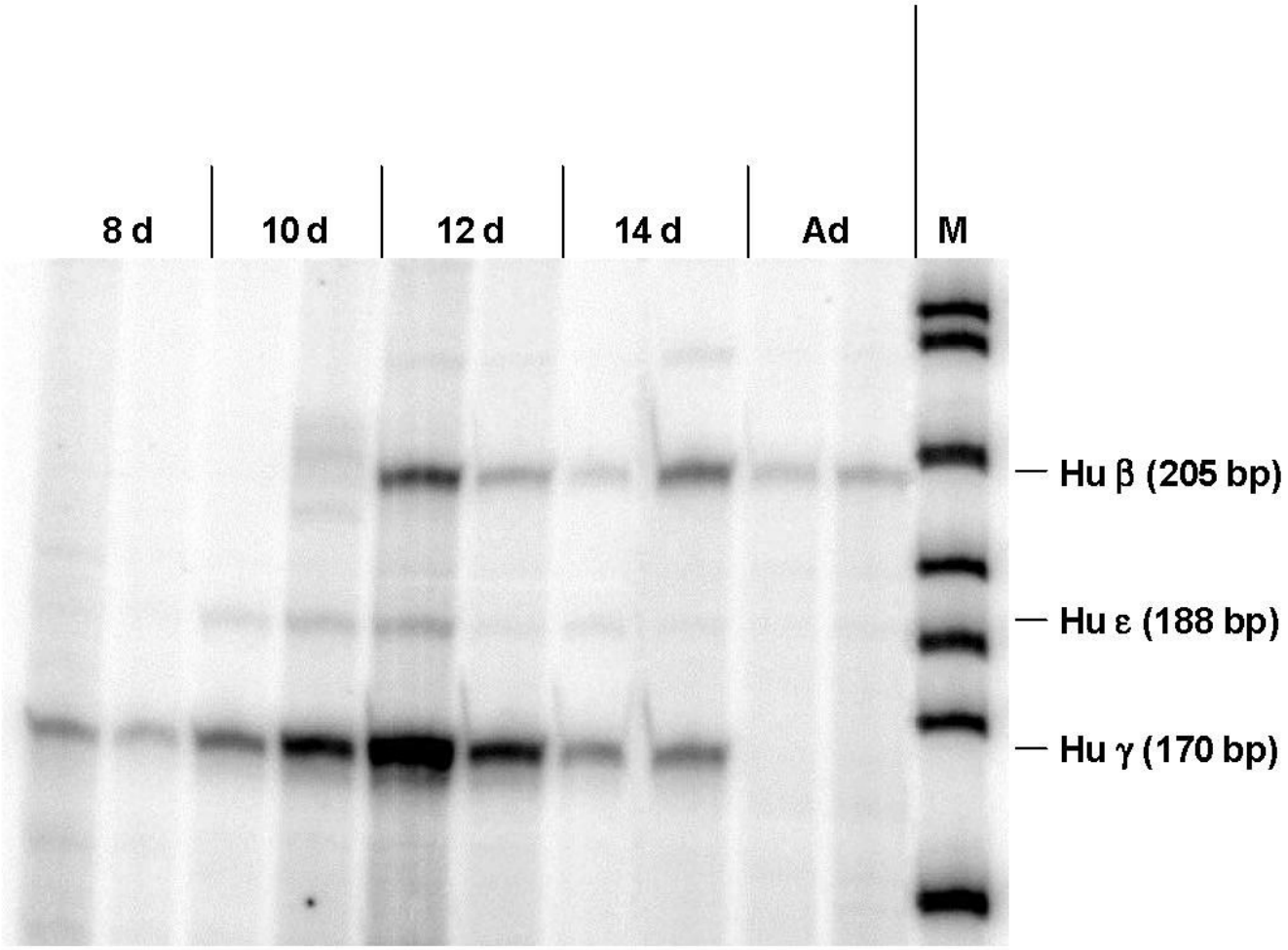

Figure 10. Temporal pattern of globin gene switching in blood of Δ5'HS1 β-YAC transgenic mice
Labeling is the same as for Figure 5. A decrease in ε-globin synthesis was observed.

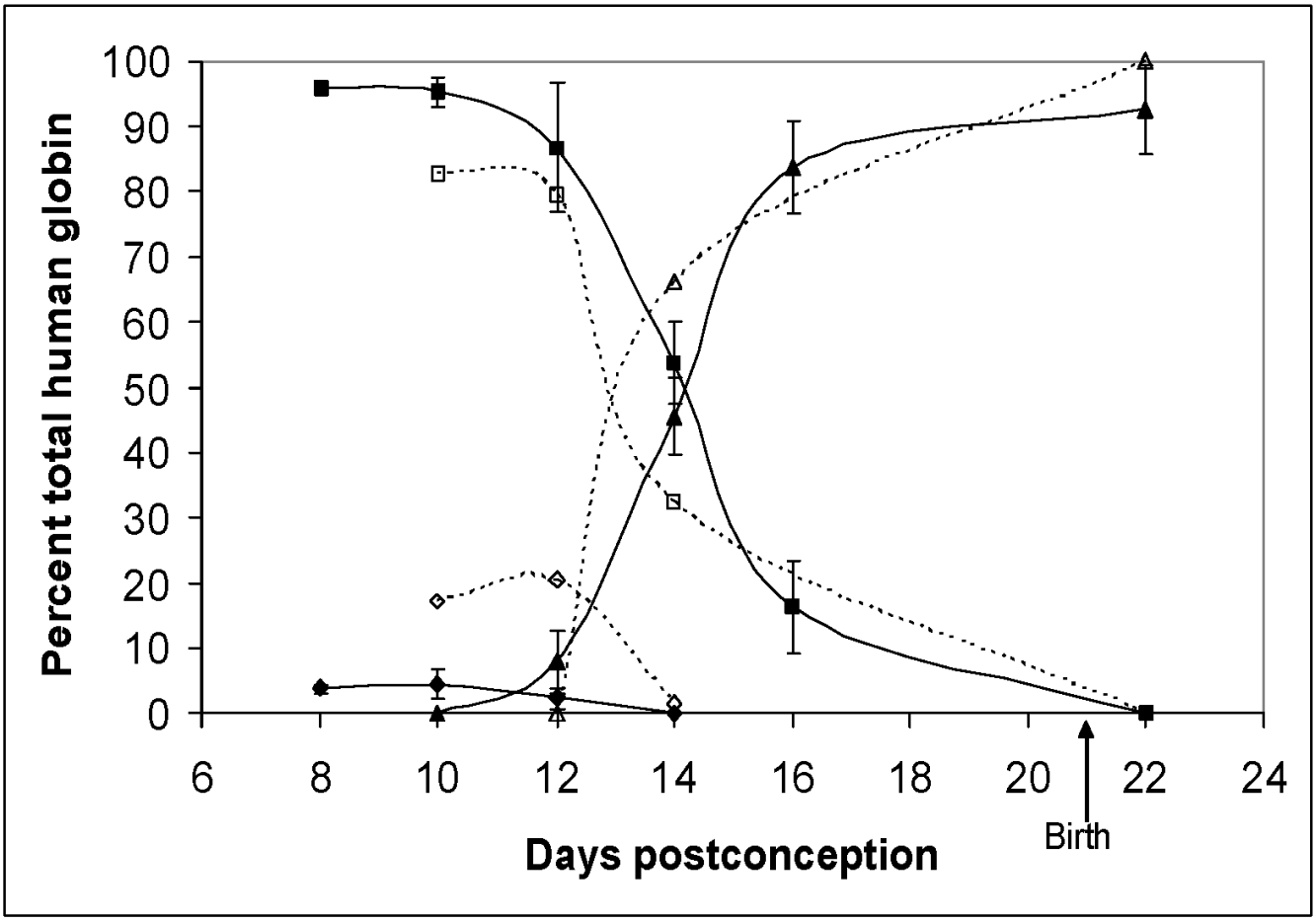

Figure 11. Globin gene switching during development in Δ5'HS1 β-YAC lines

Labeling is as for Figure 7. Experimental replicates are also the same, except that only one experiment was performed for the day 8 data. However, it included at least duplicate embryonic samples for each of the three lines. Note the decreased ε-globin gene mRNA level and the slight delay in the γ- to β-globin switch in the Δ5'HS1 lines.

Table 1

Human globin mRNA levels per copy of transgene and copy of endogenous murine α- and ζ-globin in Δ5'HS4 β-YAC transgenic mice and wild-type β-YAC control mice.

	Percentage of murine α- plus ζ-globin mRNA (mean ± SD):						
	ε-globin mRNA during embryonic erythropoiesis	γ-globin mRNA during embryonic erythropoiesis	γ-globin mRNA during definitive erythropoiesis		β-globin mRNA during definitive erythropoiesis		
βYAC line	**Day 10 yolk sac**	**Day 10 yolk sac**	**Day 12 liver**	**Day 14 liver**	**Day 12 liver**	**Day 14 liver**	**Adult blood**
Δ6	14.3±3.6	37.3±0.2	35.5±2.5	20.0±8.4	37.7±3.3	60.4±20.3	99.5±8.0
Δ35	17.8±5.6	31.4±1.1	31.0±12.0	12.0±4.1	32.2±3.4	45.3±1.8	78.0±6.4
Δ38	12.8±2.6	67.9±21.2	31.0±0.4	40.8±4.7	28.7±16.5	86.9±5.7	107.2±8.2
Mean Δ5'HS4 lines	15.0±3.9	45.5±7.5	32.5±4.9	24.3±5.7	32.9±7.7[A]	64.2±9.3	94.9±7.5
Mean wt lines	10.7±7.1	44.4±12.3	22.3±3.1	14.7±8.0	57.2±3.7[A]	65.3±10.6	116.0±11.1

Lines Δ6, Δ35 and Δ38 have two intact copies of the β-globin locus.

wt: wild-type *Ppo155* β-YAC transgenic line 26223 [47]

[A] Statistically significant difference between data pairs ($P < 0.05$)

Table 2

Human globin mRNA levels per copy of transgene and copy of endogenous murine α- and ζ-globin in Δ5'HS1 β-YAC transgenic mice and wild-type β-YAC control mice.

β-YAC line	Percentage of murine α- plus ζ-globin mRNA (mean ± SD): ε-globin mRNA during embryonic erythropoiesis Day 10 yolk sac	γ-globin mRNA during embryonic erythropoiesis Day 10 yolk sac	γ-globin mRNA during definitive erythropoiesis Day 12 liver	γ-globin mRNA during definitive erythropoiesis Day 14 liver	β-globin mRNA during definitive erythropoiesis Day 12 liver	β-globin mRNA during definitive erythropoiesis Day 14 liver	β-globin mRNA during definitive erythropoiesis Adult blood
Δ50	0.6±0.3	53.2±8.7	5.0±0.3	3.2±0.9	24.9±5.0	37.3±8.1	41.1±0.8
Δ92	1.6±0.4	61.3±20.6	11.0±4.6	3.8±2.2	38.4±12.4	37.2±17.2	91.4±47.2
Δ62	8.9	42.3	9.5±0.8	1.3±0.2	N.A.	N.A.	N.A.
Mean Δ5'HS1 lines[A]	1.1±0.4[B]	57.3±14.7	8.0±2.5[B]	3.5±1.6[B]	31.7±8.7	37.3±12.3	66.3±24.0
Mean wt lines	17.9±4.4[B]	47.9±14.1	29.9±3.7[B]	19.2±3.2[B]	38.0±4.7	57.5±16.5	113.3±13.5

Line Δ50 has 2 intact copies of the β-globin locus, Δ92 has 5 intact copies, Δ62 does not have any intact copies (see text for details).

wt: wild-type *Ppo155* β-YAC transgenic line 26223 [47]

N.A.: Not applicable

[A] Excluding line Δ62

[B] Statistically significant difference between data pairs ($P < 0.05$)