Targeted deletion of 5'HS2 of the murine β -globin LCR reveals that it is not essential for proper regulation of the β -globin locus

Steven Fiering,¹ Elliot Epner,^{1,4} Kirsten Robinson,¹ Yuan Zhuang,¹ Agnes Telling,¹ Ming Hu,⁵ David I.K. Martin,^{1,3} Tariq Enver,⁵ Timothy J. Ley,⁶ and Mark Groudine^{1,2,7}

¹Fred Hutchinson Cancer Research Center, Seattle, Washington 98104 USA; Departments of ²Radiation Oncology, ³Pediatrics, and ⁴Medicine, University of Washington School of Medicine, Seattle, Washington 98195 USA; ⁵Leukemia Research Centre at the Institute of Cancer Research, Chester Beatty Laboratories London, SW3 6JB UK; ⁶Departments of Medicine and Genetics, Jewish Hospital at Washington University Medical Center, St. Louis, Missouri 63110 USA

The β -globin locus control region (LCR) is a complex regulatory element that is essential for the appropriate red cell-specific expression of all *cis*-linked β -globin genes. Of the five hypersensitive sites that define the LCR, only 5'HS2 has been shown to augment gene expression *in vitro* in both transient and stable assays, as well as in transgenic mice. Thus, 5'HS2 has been assumed to be an important element for the function of the LCR *in vivo*. We have utilized homologous recombination in murine embryonic stem (ES) cells and phenotypic analysis in derived mice to investigate the function of 5'HS2 in its normal chromosomal position in the murine β -globin locus. Replacement of 5'HS2 with a selectable marker gene (Δ HS2+neo) causes a 2–5-fold reduction in expression of all of the genes in the locus, and a more pronounced effect (10–12-fold) on the most 5' embryonic globin gene, *Ey*, when expression of this gene is first detectable during embryogenesis. The mutation produces no alterations in the developmental timing of expression of the globin genes. When homozygous, the deletion/replacement mutation is lethal *in utero*, with the embryos dying during the stage of yolk sac and early fetal liver erythropoiesis. To distinguish phenotypic effects resulting from the deletion of 5'HS2 from those attributable to insertion of the selectable marker, the selectable marker was removed by expressing the FLP site-specific recombinase in ES cells harboring the homologous recombination event. Mice derived from these ES cells (Δ HS2 Δ neo) demonstrated nearly full expression of all the β -like globin genes on the mutated chromosome. These results indicate that although 5'HS2 demonstrates significant regulatory activities in a variety of assays, deletion of this element from the endogenous β -globin locus has no significant effect on the timing or extent of expression of the locus. In addition, this result emphasizes that when using homologous recombination to analyze complex regulatory elements *in vivo*, the inserted selectable marker must be removed to avoid influencing the phenotype of the mutation.

[*Key Words*: Thalassemia; gene targeting; locus control region; β -globin; FLP recombinase; 5'HS2]

Received June 7, 1995; revised version accepted August 1, 1995.

The β -globin locus has been studied extensively as a model of a developmentally regulated multigene locus. In a variety of vertebrate species, each of the genes in the locus is transcribed only in erythroid cells and at a specific developmental stage. The β -globin locus control region (LCR) was recognized initially as a series of DNase I hypersensitive sites (5'HS1–5) located 6–22 kb 5' of the ϵ -globin gene in the human β -globin locus (Fig. 1A) (Tuan et al. 1985; Forrester et al. 1986). 5'HS1–4 in the endogenous locus are generally considered to be erythroid specific and developmentally stable (Tuan et al.

1985; Forrester et al. 1986, 1987; Grosveld et al. 1987). However, 5'HS2 has been documented in nonhematopoietic cell lines and 5'HS5 is found in a wide variety of cell lines regardless of lineage (Dhar et al. 1990). The first indication that this region was an important *cis*-regulatory element was the observation that naturally occurring deletions of the region were associated with failure to activate any of the intact *cis*-linked β -like globin genes (Curtin et al. 1985; Taramelli et al. 1986; Driscoll et al. 1989). Subsequent to the discovery of the hypersensitive sites, it was shown that the LCR conferred high-level and position-independent expression to a linked β -globin gene in transgenic mice (Grosveld et al. 1987). Earlier studies of β -globin gene expression in

⁷Corresponding author.

Figure 1. Map of human and murine β -globin loci. (A) Human locus including the region deleted in the Hispanic thalassemia, and the insertion site of Friend-neo reported previously (Kim et al. 1992). (B) Mouse diffuse haplotype including the region deleted in this study. (C) Mouse single haplotype.

transgenic mice had demonstrated extensive variation in expression among transgenic lines related to the site of integration of the construct (position effects) (Magram et al. 1985; Townes et al. 1985).

Characterization of a naturally occurring human β -globin thalassemia, (Hispanic, Fig. 1A; Driscoll et al. 1989), in which most of the LCR was deleted, provided evidence that the LCR is essential for more than transcription (Forrester et al. 1990). In the heterozygous patient, the allele harboring the LCR deletion was DNase I resistant and late replicating in erythroid cells, as well as transcriptionally silent. In contrast, the intact locus was DNase I sensitive, early replicating, and transcribed in erythroid cells. Comparison of the deleted locus and the wild-type locus demonstrated that the effect on chromatin structure extended over 100 kb, and the alteration in replication timing extended over 200 kb. This result suggests that the LCR plays an important role in control of transcription, replication, and general chromatin structure.

The human β -globin LCR and its component DNase I hypersensitive sites have been the subject of extensive analysis, utilizing a variety of assays for effects on expression of linked genes (Grosveld et al. 1987; Forrester et al. 1989; Ryan et al. 1989; Tuan et al. 1989; Enver et al. 1990; Moi and Kan 1990; and many others; for review, see Epner et al. 1992). These experiments have assessed the influence of various LCR-based constructs on transcription, either transiently in cell lines or stably in cell lines or transgenic mice, with results summarized as follows. (1) The LCR and individual sites exhibit much higher enhancer activity in erythroid cells than in other cell types (Blom van Assendelft et al. 1989; Forrester et al. 1989; Tuan et al. 1989; Moi and Kan 1990; Ney et al. 1990). (2) The full LCR and 5'HS2 are functionally similar; they act as classic enhancers in transient assays,

increase the number of expressing clones when stably integrated in cell lines and give position independence and high-level expression in transgenic mice (Blom van Assendelft et al. 1989; Forrester et al. 1989; Ryan et al. 1989; Talbot et al. 1989; Tuan et al. 1989; Moon and Ley 1990; Sorrentino et al. 1990; Liu et al. 1992). (3) 5'HS3 and 5'HS4 have weak or no enhancer activity in transient assays but do show activity in colony assays (5'HS3) when stably integrated or in transgenic mice where they confer position independence (Tuan et al. 1987, 1989; Fraser et al. 1990; Pruzina et al. 1991; Hug et al. 1992). (4) Neither 5'HS1 nor 5'HS5 has appreciable stimulatory activity in these transfection/transgenic assays (Tuan et al. 1987; Collis et al. 1990; Fraser et al. 1993; S. Fiering et al., unpubl.). (5) Any activity associated with the LCR is stronger in the entire LCR than in any individual component, suggesting interaction or additive effects among the individual sites (Curtin et al. 1989; Forrester et al. 1989; Ryan et al. 1989; Talbot et al. 1989; Moon and Ley 1991). (6) Although the LCR is not the sole determinant of the stage-specific expression of the β -like globin genes (Enver et al. 1989; Behringer et al. 1990), it may be an important codeterminant (for review, see Engel 1993).

Although these experiments have produced valuable information about the function of the LCR, regulation of endogenous genes is accomplished in defined chromosomal locations, and it is possible that analyses performed on unintegrated or randomly integrated constructs may not reveal some important functions or may overestimate or underestimate some activities. This is particularly true of the β -globin LCR, because the region it influences is very large and has such complex regulation. To examine how the LCR components interact to regulate the endogenous β -globin locus throughout normal mammalian development, we mutated the murine LCR in embryonic stem (ES) cells using homologous recombination (HR) to derive mutant mice.

The transgenic mouse has been used extensively as a system in which to study regulation of the human β -globin locus, implying considerable similarity in functional regulation between the two species. The murine and human β -globin loci and LCRs are very similar in structure (Fig. 1), sequence, and activity (Moon and Ley 1990; Jimenez et al. 1992a). The murine and human 5'HS1–4 all exhibit >60% identity in core regions (Moon and Ley 1990; Hug et al. 1992; Jimenez et al. 1992a). The murine 5'HS5 has been identified by DNase I sensitivity but has not yet been sequenced (S. Fiering, unpubl.). The subject of this study, murine 5'HS2, contains an 800-bp region with >70% identity to the human 5'HS2, and within this large region of homology is a 60-bp core with >95% identity between human and mouse.

We report here the results of experiments in which we have deleted murine 5'HS2 and replaced it with a phosphoglycerate kinase promoter driving the neomycin phosphotransferase gene (PGK-neo)-selectable marker gene (Δ HS2+neo). This mutation results in a significant decrease in expression of all β -like globin genes, and homozygous mice derived from these mutant cells demon-

strate an embryonic lethal β -thalassemia. However, we have shown previously that the simple insertion of a selectable marker into the native LCR can severely perturb expression of the linked β -globin gene (Kim et al. 1992; Fiering et al. 1993). Thus, to determine whether the embryonic lethal phenotype was attributable to the deletion of 5'HS2, we utilized the site-specific recombinase FLP to delete the selectable marker in ES cells following the HR event. In contrast to the Δ HS2+neo mice, mice derived from these cells (Δ HS2 Δ neo) are viable and demonstrate only a minor reduction in expression of the adult β -globin genes. Thus, 5'HS2 is not required for the correct developmental timing or high-level expression of the β -like globin genes. These results emphasize the importance of analyzing regulatory elements in the context of their normal chromosomal location, as well as the importance of removing the selectable marker gene when using HR to determine the function of complex regulatory elements.

Results

Production of mice with a selectable marker replacing 5'HS2

Employing techniques described previously (Soriano et al. 1991), we have used HR to replace the 5'HS2 in the murine β -globin LCR with a PGK-neo-selectable marker (Δ HS2+neo) (Fig. 2). The 5'HS2 deletion is 1.1 kb in size and eliminates all elements shown to have activity in the various assays of LCR function described above (see introductory section). G418-resistant ES clones were screened by PCR, and positive clones were analyzed by Southern blot as shown in Figure 2F, lane 2. Additional Southern analyses with various restriction digest and probe combinations confirmed that appropriate HR events had occurred (data not shown).

Targeted clones were derived at a frequency of 1 in 60 (10 in 630) G418-resistant clones. Multiple clones were injected into blastocysts and transferred into pseudopregnant females, and two germ-line chimeras were derived. A Southern analysis demonstrating germ-line transmission is shown in Figure 2F. The data presented here are from one of the Δ HS2+neo transgenic lines. The phenotype of mice derived from either cell line was similar.

Homozygous Δ HS2+neo mutant mice die in utero

Heterozygous mice were born at expected Mendelian frequencies. However, no homozygous mutants were born among 75 live F₂ mice; homozygous mutants die in utero generally before embryonic day 12.5 (E12.5). The age of death is not well synchronized developmentally and varies between E10.5 and E13.5. Figure 3, A and B, shows E11.5 homozygous mutant and wild-type mice. The mutants are growth retarded, with pallor and often visibly enlarged hearts (cardiomegaly). These symptoms are predictable consequences of severe anemia. Hetero-

Figure 2. Modifications to the murine LCR. 5'HS sites are denoted by numbered vertical arrows. Restriction enzyme sites: (P) *Pst*I; (B) *Bam*HI; (K) *Kpn*I. (neo) Neomycin phosphotransferase controlled by PGK promoter; (HSVTK) herpes simplex virus thymidine kinase gene. (A) Murine diffuse locus; (B) Large-scale map of the 5'HS2 region; (C) HR construct; (D) modification of the LCR by HR event; (E) post-HR modification by FLP-mediated deletion of PGK-neo; (F) Southern analysis of HR events and mice derived from the HR-modified ES cells using DNA cut with *Pst*I and probed with the fragment noted in B. (Lane 1) Wild-type ES cells; (lane 2) ES cells with a HR in one allele; (lane 3) DNA from C57BL/6/J mice demonstrating the single allele; (lane 4) ES cell derived mouse with one mutant allele and one single allele; (lane 5) E12.5 embryo homozygous for the mutant allele.

zygous mutants show no gross morphological effects of the mutation at any developmental stage.

We collected blood from wild-type, heterozygous and homozygous mutant (Δ HS2+neo) embryos and examined the morphology of erythroid cells (Fig. 3C-E). At E12.5, erythrocytes of wild-type embryos display the condensed nucleus, well-hemoglobinized cytoplasm, and rounded shape typical of primitive erythrocytes. Erythrocytes from heterozygous embryos are also well hemoglobinized, but they are irregularly shaped, and immature erythroid cells are more common in these embryos. Homozygous Δ HS2+neo mutant embryos had

Figure 3. E11.5 embryos and erythrocytes of Δ HS2 + neo mice. (A,B) (Left) Embryo is homozygous for the Δ 2 + neo mutation; (right) homozygous wild type. (A) yolk sac; (B) yolk sac removed to reveal the embryo. (C–E) Wright–Giemsa-stained slides of blood from E12.5 embryo yolk sacs. (C) Wild-type; (D) heterozygous mutant; (E) homozygous mutant.

few erythroid cells at E12.5. These were almost entirely immature forms with uncondensed nuclei and irregular shapes. Hemoglobinization was poor, but many cells exhibit hemoglobinized inclusions. This morphology is characteristic of ineffective erythropoiesis seen in the marrow of humans with thalassemia major and is caused by severe deficiency of β -globin chains and associated excess of α -globin chains. Occasional primitive erythrocytes with nearly normal morphology were seen in the homozygotes. Definitive erythrocytes from peripheral blood of adult heterozygous Δ HS2 + neo mutants show mild variation in size and shape (anisopoikilocytosis) but are otherwise normal. As noted above, the homozygous Δ 2 + neo embryos die before the development of appreciable numbers of definitive erythrocytes.

The phenotype of the homozygous Δ HS2 + neo embryos indicates that the Δ HS2 + neo mutation substantially impairs expression of at least some of the β -like globin genes. Because the homozygous mutants die in utero, a complete analysis of β -like globin gene expression could only be performed in mice heterozygous for the mutation. Therefore, we developed an assay to ana-

lyze expression of each of the β -like globin genes from each allele in mutant heterozygous mice.

Assays to quantitate expression in heterozygotes

To quantitate expression accurately in heterozygous mutant mice we have exploited the existence of two distinct β -globin haplotypes in the mouse (Fig. 1). ES cells, derived from 129Sv inbred mice, contain the Hbb^D (diffuse) mouse haplotype. We crossed the germ-line chimeric mutants with the C57BL6/J strain, which carries the Hbb^S (single) β -globin haplotype. Mice obtained from this breeding are haplotype-heterozygous and have a wild-type single allele and a mutant or wild-type diffuse allele.

We have developed RT-PCR assays that exploit the difference in haplotypes in these mice to study expression of the β -like globin genes. These assays measure expression from one allele relative to expression from the other allele but are not a measure of absolute levels of mRNA. To develop these assays we identified three sets of RT-PCR primers with the following characteris-

tics: (1) One set amplifies Ey gene products, one set amplifies β h1 gene products, and one set amplifies β -globin gene products (β -major, β -minor and the single haplotype alleles, β^S and β^T); (2) each primer set amplifies the gene products from both haplotypes (i.e., the primer-binding sites are identical in both haplotypes); (3) amplified products contain restriction site differences (polymorphisms) between the haplotypes, and this allows relative quantitation by digestion with the appropriate restriction enzyme; and (4) primer sets are from adjacent exons so that amplification of DNA gives a larger band than amplification of mRNA and DNA contamination therefore can be recognized.

By analyzing the ratio of expression from the diffuse allele to expression from the single allele we can control for variation between samples and measure expression differences accurately between (wild-type diffuse/wild-type single) mice and (mutant diffuse/wild-type single) littermates. The accuracy of this assay was tested in mixing experiments in which cDNA from homozygous diffuse or single mice was combined in varying ratios and then assayed. These results show that the ratio of diffuse/single mRNA revealed by the assay is an accurate determination of the diffuse/single ratio of known input cDNA for Ey, β h1, and β -globin genes (Fig. 4).

Expression of all β -like globin genes is reduced in Δ HS2 + neo mice

Utilizing this RT-PCR assay we determined that deletion of 5'HS2 and the associated insertion of PGK-neo (Δ HS2 + neo) leads to a two- to fourfold reduction in expression of all β -like globin genes from the mutated chromosome (Fig. 5; Table 1). The only exception is the $\sim$ 10-fold reduction in Ey expression in E9.5 embryos. This exceptionally large reduction in Ey was observed in three individual litters. E9.5 is the first day of significant β -like globin gene expression, and by E10.5 the reduction in Ey expression is fourfold, similar to the two- to fourfold reduction found in all other developmental times and with all other β -like genes (including β h1 at E9.5). The impairment of expression of β -major and β -minor from the Δ 2 + neo allele was further confirmed by acid-urea gel analysis of adult peripheral blood (data not shown), in which the protein products of the diffuse haplotype (β -major and β -minor) can be distinguished from the products of the single haplotype (β^S and β^T).

To determine whether the deletion of 5'HS2 had perturbed the developmental control of the locus, we examined the timing of expression of the genes from the Δ 2 + neo locus. Specifically we assayed for expression of

Figure 4. Mixing experiments to characterize RT-PCR/restriction digest assay for quantitation of expression of β -like globin genes. RT reaction was performed on RNA extracted from single (C57BL6/J) and diffuse (129Sv) haplotype mice. Assays were done by mixing the RT reactions from mice of each haplotype in denoted amounts (RT reactions were normalized to give equal activity per volume), amplifying by PCR and digesting with restriction enzymes. The maps show the restriction enzyme used and band size derived for each assay. The ratios observed are compared to the ratios expected from the known composition of the mixes. (A) Ey gene assay, RNA from E10.5 yolk sac; (B) β h1 assay, RNA from E10.5 yolk sac; (C) β -major plus β -minor (diffuse) vs. β^S plus β^T (single) assay, RNA derived from peripheral blood.

Figure 5. Expression of β -like globin genes from Δ H52 + neo heterozygous mice. (wt) Wild-type diffuse/wild-type single mice; (Δ 2) Δ H52 + neo/wild-type single mice; (E9.5, E10.5, E11.5) Embryonic day 9.5, 10.5, and 11.5. (A) Ey assay; (B) β h1 assay; (C) β major plus β minor (diffuse) vs. β^S plus β^T (single).

Ey and β h1 in fetal liver and for expression of β -major and β -minor in yolk sac and found no perturbation in developmental control (data not shown).

Deletion of the selectable marker gene (Δ H52 Δ neo mice) restores viability

We reported previously that the simple insertion of an expressed selectable marker in the LCR severely perturbed expression of the linked β -globin gene. To analyze the phenotype of the 5'HS2 deletion without the influence of the selectable marker, we used the FLP recombinase to remove the selectable marker. The targeting vector had FLP recombinase target (FRT) sites flanking the PGK-neo gene in an orientation that would allow FLP expression to mediate a deletion of the selectable marker (O'Gorman et al. 1991). This target site-specific reaction leaves a single FRT site (48 bp) at the site of recombination. FLP was expressed transiently in the ES cell line harboring the Δ H52 + neo HR that had been used to generate the germ-line chimera whose offspring were analyzed above. The FLP protein was expressed from a bicistronic message containing FLP, an internal ribosome entry site and the *lacZ* gene as utilized previously (Fiering et al. 1993). A pool of cells expressing *lacZ* was selected by FACS/Gal sorting (Nolan et al. 1988), and clones derived from this pool were tested for G418 sensitivity.

Approximately 10% of these clones were G418 sensi-

tive. G418 sensitive clones (90%) were determined by Southern blot to have an FLP mediated deletion of the PGK-neo marker. A germ-line chimera was derived from one of these Δ H52 Δ neo clones (Fig. 6), and expression of the β -like globin genes was determined throughout development, as described above for the Δ H52 + neo mutation.

Removal of the PGK-neo marker restored most of the β -like gene expression (Fig. 7; Table 2). β -Globin expression is $\sim$ 70% of wild-type expression, and Ey expression is indistinguishable from wild type. β h1 appears to be slightly impaired at E10.5 and E11.5; however, because some mice assayed on those days showed no impairment at all relative to wild type, we conclude that it is unimpaired or very mildly impaired. In addition, expression of Ey in E9.5 embryos is no different than expression of Ey at E10.5 and E11.5, in contrast to the extreme suppression of E9.5 Ey expression in Δ H52 + neo mice. Mice homozygous for the Δ H52 Δ neo mutation are viable and are born at expected Mendelian frequencies. Examination of their peripheral blood shows no significant cellular abnormality. As discussed above for the Δ H52 + neo mice, there is no perturbation of developmental regulation in the Δ H52 Δ neo mice.

Discussion

5'HS2 is not required for proper developmental timing or near normal expression levels of the β -like globin genes

To examine the function of 5'HS2 in its normal chromosomal context, we have used homologous recombination to delete 5'HS2 (and FLP recombinase to delete the inserted selectable marker) from the murine LCR (Δ H52 Δ neo). As discussed in the introductory section, 5'HS2 of the human β -globin LCR has been studied extensively in cell lines and transgenic mice. Taken to-

Table 1. Ratio of diffuse allele expression to single allele expression for Δ H52 + neo mice

Age	WTD/WTS ratio ^a	Δ 2 + neo/WTS ratio ^b
<i>Ey</i>		
E9.5	1.06 (S.D. 0.08, n = 7)	0.11 (S.D. 0.03, n = 12)
E10.5	1.15 (S.D. 0.07, n = 7)	0.25 (S.D. 0.02, n = 4)
E11.5	1.14 (S.D. 0.02, n = 6)	0.38 (S.D. 0.01, n = 5)
<i>βh1</i>		
E9.5	0.98 (S.D. 0.15, n = 8)	0.43 (S.D. 0.04, n = 8)
E10.5	0.87 (S.D. 0.04, n = 7)	0.30 (S.D. 0.04, n = 4)
E11.5	0.82 (S.D. 0.04, n = 6)	0.33 (S.D. 0.07, n = 5)
<i>β major + β minor</i>		
E15.5	0.97 (S.D. 0.02, n = 3)	0.28 (S.D. 0.04, n = 2)
E17.5	0.93 (S.D. 0.05, n = 5)	0.33 (S.D. 0.03, n = 4)
Adult	1.10 (S.D. 0.15, n = 6)	0.35 (S.D. 0.07, n = 6)

^a(WTD/WTS) Ratio of expression from the wild-type diffuse allele to expression from the wild-type single allele.

^b Δ 2 + neo/WTS) Ratio of expression from the Δ H52 + neo diffuse allele to expression from wild-type single allele.

Figure 6. Southern blot of the Δ HS2 Δ neo mutant cells and mice. Probe is shown in Fig. 2B. DNA was digested with *Bam*HI (see Fig. 2). (Lane 1) Wild-type ES cells; (lane 2) mutant ES cells with Δ HS2 + neo mutation; (lane 3) C57BL6/J mouse DNA (homozygous single); (lane 4) ES cell line with Δ HS2 Δ neo mutation after FLP expression; (lane 5) heterozygous mutant mouse (Δ HS2 Δ neo/wild-type single) derived from ES cells assayed in lane 4; (lane 6) homozygous adult Δ HS2 Δ neo mutant mouse.

gether, the previous studies suggest that 5'HS2 plays an important role in LCR function. Surprisingly, our experiments show that the β -globin locus functions almost normally without 5'HS2. Deletion of 5'HS2 has no effect on the developmental timing of expression of any of the β -like globin genes, has little or no effect on expression levels of Ey or β h1, and causes only a small (30%) reduction in expression level of β -major and β -minor.

To have confidence in this conclusion it is important to ascertain that we have deleted all of the functional

Table 2. Ratio of diffuse allele expression to single allele expression for Δ HS2 Δ neo mice

Age	WTD/WTS ratio ^a	Δ 2 Δ neo/WTS ratio ^b
<i>Ey</i>		
E9.5	1.06 (s.d. 0.08, <i>n</i> = 7)	1.13 (s.d. 0.07, <i>n</i> = 5)
E10.5	1.15 (s.d. 0.07, <i>n</i> = 7)	1.14 (s.d. 0.03, <i>n</i> = 4)
E11.5	1.14 (s.d. 0.02, <i>n</i> = 6)	1.01 (s.d. 0.13, <i>n</i> = 5)
β h1		
E9.5	0.98 (s.d. 0.15, <i>n</i> = 8)	1.05 (s.d. 0.08, <i>n</i> = 5)
E10.5	0.87 (s.d. 0.04, <i>n</i> = 7)	0.74 (s.d. 0.03, <i>n</i> = 3)
E11.5	0.82 (s.d. 0.04, <i>n</i> = 6)	0.69 (s.d. 0.05, <i>n</i> = 3)
β major + β minor		
E15.5	0.97 (s.d. 0.02, <i>n</i> = 3)	0.73 (s.d. 0.01, <i>n</i> = 2)
E17.5	0.93 (s.d. 0.05, <i>n</i> = 5)	0.82 (s.d. 0.12, <i>n</i> = 8)
Adult	1.10 (s.d. 0.15, <i>n</i> = 6)	0.65 (s.d. 0.04, <i>n</i> = 5)

^a(WTD/WTS) Ratio of expression from the wild-type diffuse allele to expression from the wild-type single allele.

^b Δ 2 Δ neo/WTS) Ratio of expression from the Δ HS2 Δ neo diffuse allele to expression from wild-type single allele.

sequences of the 5'HS2 site. The 1.1-kb *Eco*RI fragment used in the functional analysis of the murine 5'HS2 site contains 800 bp with >70% identity to the human 5'HS2. The *Eco*RI sites that define this fragment are contained within two direct repeats that flank the murine 5'HS2 region. These repeats contain *Kpn*I sites adjacent to the *Eco*RI sites, and this 1.1-kb *Kpn*I fragment was deleted in the HR construct. Thus, the region deleted in our experiments is virtually identical to the fragment originally used to define the functional murine 5'HS2 region (Moon and Ley 1990). This fragment has been shown to be functionally equivalent to human 5'HS2. It acts as an enhancer in transient assays or when stably integrated in cell lines (Moon and Ley 1990) and confers high level position-independent erythroid-specific expression when linked to the human β -globin gene in transgenic mice (Enver et al. 1994). Finally, we have assayed the Δ HS2 Δ neo mice for hypersensitivity of the remaining DNA in the region of 5'HS2. These assays were performed in erythroblasts isolated from spleens of phenylhydrazine-treated mice and showed no evidence of DNase I hypersensitivity in the region of the deletion but did show normal formation of 5'HS1 and 5'HS3 (data not shown).

Whereas the human 5'HS2 has been analyzed in detail to define the precise sequences that are effective in the various functional assays used previously to study the LCR, the murine 5'HS2 has not been studied in such detail. However, our 5'HS2 deletion did eliminate the elements in the murine locus homologous to the primary active elements in the human 5'HS2. These include duplicated AP-1 consensus-binding sites that bind NF-E2 in vitro (Talbot and Grosfeld 1991). These NF-E2 sites are located in the core region of 95% murine/human homology and have been shown to be necessary and sufficient for the enhancer activity of human 5'HS2 (Caterina et al. 1991; Liu et al. 1992; Caterina et al. 1994) and for the ability of human 5'HS2 to induce linked

Figure 7. Expression of β -like globin genes from Δ HS2 Δ neo heterozygous mice. (wt) Wild-type diffuse/wild-type single mice; (Δ 2) Δ HS2 Δ neo/wild-type single mice. (E9.5, E10.5, E11.5) Embryonic day 9.5, 10.5, and 11.5. (A) Ey assay; (B) β h1 assay; (C) β -major plus β -minor (diffuse) vs. β^S plus β^T (single).

genes during terminal erythroid differentiation of mouse erythroleukemia (MEL) cells (Talbot and Grosveld 1991). The human 5'HS2 has been recognized as a collection of individual sites dispersed over 600 bp (Talbot et al. 1990), and our murine 5'HS2 deletion eliminates the entire region homologous to this 600-bp fragment. In addition, the deletion almost entirely overlaps the region homologous to the human 732-bp *HindIII*–*BglII* fragment that has been shown to be functionally similar to larger 5'HS2 fragments in transgenic analyses (Caterina et al. 1994).

Implication of the Δ HS2 Δ neo phenotype for models of LCR function

Two general models of LCR function have been considered. One model proposes that the individual 5'HS sites work additively or synergistically to establish and maintain the chromatin structure of the β -globin locus in a conformation permissive for β -like globin gene expression in erythroid cells. This model predicts that the phenotype resulting from deletion of any individual 5'HS would be mild and would not demonstrate stage- or gene-specific effects. Deletions of multiple 5'HSs, however, would result in defects in gene expression at all stages and in all genes, and when sufficient numbers of 5'HSs are deleted, the entire chromatin structure would be altered and resemble the conformation in nonerythroid cells (as in the thalassemias that delete most or all of the LCR; Curtin et al. 1985; Taramelli et al. 1986; Driscoll et al. 1989).

The second model proposes that individual 5'HS sites control the expression of individual genes at specific developmental stages. This model predicts that deletion of individual sites will have severe effects on particular genes and/or particular stages. The models are not mutually exclusive and the LCR may be a functional hybrid of the two models. For example, individual 5'HSs may work cooperatively to alter the topology of the locus but individually to control the expression of specific genes in the locus. As discussed below, some support for both models is found in the phenotype associated with deletion of 5'HS2.

5'HS2 is the only 5'HS site with the functional properties of the entire LCR, and it is hypersensitive to DNase I in multipotential hematopoietic progenitor cells prior to differentiation (Jimenez et al. 1992b). These observations led to the suggestion that 5'HS2 may be required to set up the appropriate chromatin structure of the locus. However, we have demonstrated that 5'HS2 can be deleted without grossly impairing the function of the β -globin locus, suggesting that 5'HS2 has no unique function in establishing the structure of the locus. These data supports the first, "general activity," model. Consistent with this, *in vitro* and *in vivo* footprinting studies of the LCR have suggested that a small number of binding motifs are bound by proteins and that each of the individual hypersensitive sites has basically the same protein binding sites, although in different amounts and with different spatial arrangements [for review, see Crossley and Orkin 1993]. The sites generally recognized

are for GATA-1, AP-1/NF-E2, and CACC binding proteins (possibly SP1 and/or EKLf). As proposed previously (Forrester et al. 1989), the high concentration of factor-binding sites in the LCR may serve to displace nucleosomes or other chromatin-binding proteins, resulting in the stable alteration of topology of the β -globin locus in erythroid cells. In this context, deletion of individual 5'HSs may not delete sufficient numbers of binding sites to significantly alter the topology of the locus.

Previous transfection and transgenic experiments also support the idea that the individual 5'HS sites contribute additively (or synergistically) to the general function of the LCR in controlling expression of the locus. For example, constructs with single 5'HS sites demonstrate a significant portion of the activity of constructs containing 5'HS1–4 (Curtin et al. 1989; Forrester et al. 1989; Ryan et al. 1989; Talbot et al. 1989; Moon and Ley 1991). In addition, the basic definition of an LCR, providing high-level position-independent expression regardless of transgene integration site has been satisfied for 5'HS2–4 individually, suggesting that at least each of these three sites is performing similar functions.

The second model has been formulated explicitly in a proposal that 5'HS2–4 of the LCR physically associate with and control individual β -like globin genes and, in this way, influence globin gene switching (Engel 1993). Because the deletion of 5'HS2 does not alter the stage-specific or high-level expression of any of the β -like globin genes, our data appear to conflict with this model. However, our finding that deletion of 5'HS2 does reduce expression of the β -globin genes slightly (without altering *Ey* or β h1 expression) implies that elements in the deleted region may influence expression of specific genes. It is possible that 5'HS2 may have unique gene-specific functions in the intact LCR, but other 5'HS sites can perform these functions when 5'HS2 is deleted. Analogous situations have been documented in experiments in which structural genes are deleted. For example, deletion of either *MyoD* or *Myf5* does not affect skeletal muscle development, implying that neither gene has a unique function in skeletal myogenesis. However, the loss of *MyoD* does lead to an increased level of *Myf5* expression and the loss of both genes blocks development of skeletal muscle completely (Rudnicki et al. 1993).

The demonstration that deletion of an LCR hypersensitive site causes very little change in expression of the locus may recur as other hypersensitive sites are deleted individually in future experiments. More dramatic alterations in overall expression of the locus may require deletion of several 5'HSs, as in the $(\gamma\delta\beta)^0$ thalassemias. We should emphasize however, that the lack of a strong stage-specific effect of the 5'HS2 deletion does not imply that deletion of other individual hypersensitive sites, such as 5'HS3 or 5'HS4, will not have stage-specific effects. Further experiments will be required to determine whether deletion and/or replacement of combinations of hypersensitive sites influence stage- and gene-specific expression or whether all such combinations affect only the general activity of the locus.

Implications of the Δ HS2 + neo mice

The phenotype of the 5'HS2 deletion and concomitant insertion of the PGK-neo selectable marker (Δ HS2 + neo) is severe; all β -like genes are reduced in level of expression by two- to four-fold (except Ey in the first day of erythropoiesis; see below), and the mutation is lethal when homozygous. This result is similar to our previous finding that insertion of a Friend retrovirus long terminal repeat (LTR)-driven selectable marker gene into the human LCR between 5'HS1 and 5'HS2 (see Fig. 1A) suppressed the expression of adult β -globin in MEL cell somatic hybrids (Kim et al. 1992; Fiering et al. 1993). However, there are some major differences between those previous experiments and the results reported here. Most important of these differences is the deletion of 5'HS2. The phenotype of the Δ 2 + neo mice may be related to both the insertion of PGK-neo and the deletion of 5'HS2. The simplest interpretation of the present results is that there is competition for the LCR (without 5'HS2) between the PGK promoter and the promoters of the β -like globin genes, and that the proximity of the PGK promoter to the LCR gives it an advantage in this competition. This result supports studies suggesting that expression of adult β -globin is mediated by competition with the embryonic and fetal genes that are favored until birth (Behringer et al. 1990; Enver et al. 1990). These results also reinforce the idea that removal of the selectable marker gene is an essential component of HR analysis of transcriptional regulatory elements (Fiering et al. 1993; Cogne et al. 1994).

The very severe (10–12-fold) reduction of Ey expression in E9.5 yolk sac is reproducible and significantly different from the reduction in the other genes, including the expression of β h1 in the same samples. Ey expression is indistinguishable from wild type after removal of the PGK-neo marker. We hypothesize that this more severe impairment of Ey transcription is related to the close proximity of Ey to PGK-neo and to the presumed interaction between the PGK promoter and the LCR prior to expression of the β -like globin genes.

The severe impairment of β -like gene expression associated with the Δ HS2 + neo mutation causes an embryonic β -thalassemia, a phenotype that has not been described previously in humans or mice. Homozygous embryos have severely impaired production of the embryonic β -like globin genes, die by E13.5 with severe anemia, and have primitive erythrocytes with striking morphologic abnormalities similar to those seen in definitive erythrocytes of humans and mice with β -thalassemia.

The majority of human β -thalassemias affect production of only adult (δ and β) β -globins. The β -thalassemias are presumed to be common because of the protective effect of heterozygosity in malarial infections occurring in early childhood. An embryonic (ϵ) thalassemia would be unlikely to create such an adaptive advantage and such thalassemias have not been reported in humans. Nevertheless, this phenotype suggests the possibility of an animal model system to test various therapeutic

strategies for the in utero rescue of an embryonic lethal anemia.

Materials and methods*Production and screening of mutant mice*

Clones containing the β -globin LCR region of interest were isolated from a 129/Sv genomic library (Stratagene). The region containing HS2 is flanked by *Kpn*I sites. This 1.1-kb *Kpn*I fragment was deleted and replaced with a neomycin resistance gene expression cassette (PGKneobpA; Soriano et al. 1991). The construct had 925 bp of 3' homology and 11.3 kb of 5' homology. Linearized targeting vector (25 μ g) was electroporated into AK-7 (A. Imamoto and P. Soriano, unpubl.) ES cells and colonies that were G418 and gancyclovir resistant were isolated. The colonies were screened by PCR for homologous recombinants as described previously (Soriano et al. 1991) using the following primers: BPA [hybridizes in the poly(A) sequence of the PGK-neo gene] GATTGGGAAGACAATAGCAGGCATGC, and mush 8 [hybridizes to the murine LCR outside of the region of homology] AGACTATTCCAGGGCCTAGC. HR events were confirmed by Southern blotting. Figures containing autoradiograms used computer scans of the original X-ray films.

RT-PCR assays

RNA was isolated using RNazol (Tel-Test, Inc.) and was resuspended in TE with an aliquot diluted to 50 ng/ μ l. The reverse transcriptase reaction was performed in the following 1 $\times$ buffer A: 50 mM KCl, 1.5 mM MgCl₂, 10 mM Tris at pH 8.4, 0.01% gelatin. For a 10- μ l reaction we added the following, keeping all reagents on ice: 1 μ l of 10 $\times$ buffer A, 0.2 μ l of a 10 mM solution of each deoxynucleotide triphosphate, 0.1 ng of random hexamers, 10 units of RNasin (Promega), 200 units of Moloney RT (GIBCO/BRL), 1 μ l of 100 mM DTT, and 100–200 ng of RNA and H₂O to bring to 10 μ l. The reaction was overlaid with mineral oil and incubated at 55°C for 30 min. After the incubation the reaction was diluted with an additional 40 μ l of TE to make an RT stock solution and stored at –20°C.

PCR reactions were performed as follows. To each 25 μ l reaction was added 2.5 μ l of 10 $\times$ buffer A (see above), 0.2 μ l of a 10 mM solution of each deoxynucleotide triphosphate, 0.05–0.25 μ l of [α ³²P]dCTP (3000 Ci/mmol, we used 0.05 μ l for Ey and β -globin and 0.25 μ l for β h1), 150 ng of each primer, and 0.25 μ l of *Taq* polymerase (Perkin-Elmer), and H₂O to bring each reaction up to a volume of 24 μ l. RT stock solution is further diluted 1:10 in TE for working RT solution and 1.0 μ l of working RT solution is added to each PCR. The PCR program is one melting incubation of 95°C for 2 min followed by 21–28 cycles of 93°C for 30 sec, 63°C for 30 sec, and 72°C for 60 sec. We used 23 cycles for Ey, 28 cycles for β h1, and 21 cycles for β -globin.

The PCR products were digested with the appropriate restriction enzymes using 10 μ l of PCR product, 10–20 units of restriction enzyme, 2 μ l of the 10 $\times$ buffer appropriate for the restriction enzyme (use acetate-based buffers, as NaCl-based buffers do not run well on the gel), and H₂O, to a final volume of 20 μ l. Ey and β h1 are digested with *Xba*I, β -globin is digested with *Bst*XI (and then subsequently digested with *Pvu*I to differentiate β -major from β -minor if desired).

The products were analyzed by loading 6–10 μ l of the PCR product plus loading dye on 6% acrylamide gels (spacer 0.4 mm) and electrophoresing for 2–2.5 hr at 11 W constant power (320–400 mV, 30–40 mA). The results were visualized by overnight

autoradiography and quantitated by phosphorImaging of the dried gel.

The assay is most reproducible with low concentration of products because high concentrations of products allow formation of heteroduplexes during the last melting step in the PCR. These heteroduplexes will not cut with the restriction enzyme, as there is a 1-base mismatch on one strand. Heteroduplexes will overestimate the relative amount of the product lacking the diagnostic restriction site and give an inaccurate ratio, as demonstrated elsewhere (McCulloch et al. 1995; Schneeberger et al. 1995). To overcome this problem, care must be taken to use multiple wild-type controls on each PCR and determine that the wild-type controls have a ratio of ~1 and have approximately the same strength of signal (total counts) as the experimental samples (indicating the same concentration of products). An alternative approach to achieving consistent results is to withdraw aliquots of the PCR in intervals of two cycles and gel-analyze those samples to determine the cycle at which the ratio begins to change because of heteroduplex formation.

Ey is amplified with the following primers: Ey-1R, AACCCCTCATCAATGGCCTGTGG; and Ey-2R, TCAGTGGTACTTGTGGGACAGC, and digested with *Xba*I for >3 hr at 37°C. β h1 is amplified with the following primers: β h1-5F, CACTCGAGATCATCTCCAAGC; and β h1-5R, TAACCCCAAGCCCAAGGATG, and digested with *Xba*I as for Ey. β -major, β -minor, β^S , and β^T are amplified with the following primers: β m-4, CACCTTTGCCAGCCTCAGTG; and β m-2R, GGTTTAGTGGTACTTGTGAGCC. To compare β -major plus β -minor versus β^S plus β^T , *Bst*XI was digested with at 55°C for >3 hr.

Acknowledgments

We thank Phil Soriano for his generous help and support in the generation and detection of targeted deletions in ES cells; Andreas Reik and Michael Bender for critical reading of the manuscript; and the Hutchinson Cancer Center Image Analysis Laboratory for PhosphorImaging quantitation and assistance in preparation of the figures. This work was supported by National Institutes of Health (NIH) Fellowship (HL08732) to S.F., and NIH grants (HL48790 to D.I.K.M. DK38682 to T.J.L., and HL48356 and DK44746 to M.G.).

The publication costs of this article were defrayed in part by payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 USC section 1734 solely to indicate this fact.

References

- Behringer, R.R., R.E. Hammer, R.L. Brinster, and T.M. Townes. 1990. Human γ - to β -globin switching in transgenic mice. *Genes & Dev.* **4**: 380–389.
- Blom van Assendelft, G., O. Hanscombe, F. Grosveld, and D.R. Greaves. 1989. The β -globin dominant control region activates homologous and heterologous promoters in a tissue-specific manner. *Cell* **56**: 969–977.
- Caterina, J.J., T.M. Ryan, K.M. Pawlik, R.D. Palmiter, R.L. Brinster, R.R. Behringer, and T.M. Townes. 1991. Human β -globin locus control region: Analysis of the 5' DNase I hypersensitive site HS2 in transgenic mice. *Proc. Natl. Acad. Sci.* **88**: 1626–1630.
- Caterina, J.J., D.J. Ciavatta, D. Donze, R.R. Behringer, and T.M. Townes. 1994. Multiple elements in human β -globin locus control region 5'HS2 are involved in enhancer activity and position-independent, transgene expression. *Nucleic Acids Res.* **22**: 1006–1011.
- Cogne, M., R. Lansford, A. Bottaro, J. Zhang, J. Gorman, F. Young, H.L. Cheng, and F.W. Alt. 1994. A class switch control region at the 3' end of the immunoglobulin heavy chain locus. *Cell* **77**: 737–747.
- Collis, P., M. Antoniou, and F. Grosveld. 1990. Definition of the minimal requirements within the human β -globin gene and the dominant control region for high level expression. *EMBO J.* **9**: 233–240.
- Crossley, M. and S.H. Orkin. 1993. Regulation of the β -globin locus. *Curr. Opin. Gen. Dev.* **3**: 232–237.
- Curtin, P., M. Pirastu, Y.W. Kan, J.A. Gobert-Jones, A.D. Stephens, and H. Lehmann. 1985. A distant deletion affects β -globin gene function in an atypical $\gamma\delta\beta$ -thalassemia. *J. Clin. Invest.* **76**: 1554–1558.
- Curtin, P.T., D. Liu, W. Liu, J.C. Chang, and Y.W. Kan. 1989. Human β -globin gene expression in transgenic mice is enhanced by a distant DNase I hypersensitive site. *Proc. Natl. Acad. Sci.* **86**: 7082–7086.
- Dhar, V., A. Nandi, C. Schildkraut, and A. Skoultschi. 1990. Erythroid-specific nuclease-hypersensitive sites flanking the human β -globin domain. *Mol. Cell. Biol.* **10**: 4324–4333.
- Driscoll, C., C.S. Dobkin, and B.P. Alter. 1989. $\gamma\delta\beta$ thalassemia due to a de novo mutation deleting the 5' β -globin locus activating region hypersensitive sites. *Proc. Natl. Acad. Sci.* **86**: 7470–7474.
- Engel, J.D. 1993. Developmental regulation of human β -globin gene transcription: a switch of loyalties. *Trends Genet.* **9**: 304–309.
- Enver, T., A.J. Ebens, W.C. Forrester, and G. Stamatoyannopoulos. 1989. The human β -globin locus activation region alters the developmental fate of a human fetal globin gene in transgenic mice. *Proc. Natl. Acad. Sci.* **86**: 7033–7037.
- Enver, T., N. Raich, A.J. Ebens, T. Papayannopoulou, F. Costantini, and G. Stamatoyannopoulos. 1990. Developmental regulation of human fetal-to-adult globin gene switching in transgenic mice. *Nature* **344**: 309–313.
- Enver, T., Q. Li, K. Gale, M. Hu, G. May, J. Karlinsey, G. Jimenez, T. Papayannopoulou, and F. Costantini. 1994. Analysis of the developmental and transcriptional potentiation functions of 5'HS2 of the murine-globin locus control region in transgenic mice. *Dev. Biol.* **165**: 574–584.
- Epner, E., C.G. Kim, and M. Groudine. 1992. What does the locus control region control? *Curr. Biol.* **2**: 262–264.
- Fiering, S., C.G. Kim, E.E. Epner, and M.G. Groudine. 1993. An "in-out" strategy using gene targeting and FLP recombinase for the functional dissection of complex DNA regulatory elements: Analysis of the β -globin locus control region. *Proc. Natl. Acad. Sci.* **90**: 8469–8473.
- Forrester, W.C., C. Thompson, J.T. Elder, and M. Groudine. 1986. A developmentally stable chromatin structure in the human β -globin gene cluster. *Proc. Natl. Acad. Sci.* **83**: 1359–1363.
- Forrester, W.C., S. Takegawa, T. Papayannopoulou, G. Stamatoyannopoulos, and M. Groudine. 1987. Evidence for a locus activation region: The formation of developmentally stable hypersensitive sites in globin-expressing hybrids. *Nucleic Acids Res.* **15**: 10159–10177.
- Forrester, W.C., U. Novak, R. Gelinas, and M. Groudine. 1989. Molecular analysis of the human β -globin locus activation region. *Proc. Natl. Acad. Sci.* **86**: 5439–5443.
- Forrester, W.C., E. Epner, M.C. Driscoll, T. Enver, M. Brice, T. Papayannopoulou, and M. Groudine. 1990. A deletion of the Human β -globin locus control region causes a major alteration in chromatin structure and replication across the entire β -globin locus. *Genes & Dev.* **4**: 1637–1649.
- Fraser, P., J. Hurst, P. Collis, and F. Grosveld. 1990. DNase I

- hypersensitive sites 1,2, and 3 of the human β -globin dominant control region direct position-independent expression. *Nucleic Acids Res.* **18**: 3503–3508.
- Fraser, P., S. Pruzina, M. Antoniou, and F. Grosveld. 1993. Each hypersensitive site of the human β -globin locus control region confers a different development pattern of expression on the globin genes. *Genes & Dev.* **7**: 106–113.
- Grosveld, F., G. Blom van Assendelft, D. Greaves, and G. Kollias. 1987. Position-independent, high-level expression of the human β -globin gene in transgenic mice. *Cell* **51**: 975–985.
- Hug, B.A., A.M. Moon, and T.J. Ley. 1992. Structure and function of the murine β -globin locus control region 5' HS-3. *Nucleic Acids Res.* **20**: 5771–5778.
- Jimenez, G., K.B. Gale, and T. Enver. 1992a. The mouse β -globin locus control region: Hypersensitive sites 3 and 4. *Nucleic Acids Res.* **20**: 5797–5803.
- Jimenez, G., S.D. Griffiths, A.M. Ford, M.F. Greaves, T. Enver. 1992b. Activation of the β -globin locus control region precedes commitment to the erythroid lineage. *Proc. Natl. Acad. Sci.* **89**: 10618–10622.
- Kim, C.G., E.M. Epner, W.C. Forrester, and M. Groudine. 1992. Inactivation of the human β -globin gene by targeted insertion into the β -globin locus control region. *Genes & Dev.* **6**: 928–938.
- Liu, D., J.C. Chang, P. Moi, W. Liu, Y.W. Kan, and P.T. Curtin. 1992. Dissection of the enhancer activity of β -globin 5' DNase I-hypersensitive site 2 in transgenic mice. *Proc. Natl. Acad. Sci.* **89**: 3899–3903.
- Magram, J., K. Chada, and F. Costantini. 1985. Developmental regulation of a cloned adult β -globin gene in transgenic mice. *Nature* **315**: 338–340.
- McCulloch, R.K., C.S. Choong, and D.M. Hurley. 1995. An evaluation of competitor type and size for use in the determination of mRNA by competitive PCR. *PCR Methods Applic.* **4**: 219–226.
- Moi, P. and Y.W. Kan. 1990. Synergistic enhancement of globin gene expression by activator protein-1-like proteins. *Proc. Natl. Acad. Sci.* **87**: 9000–9004.
- Moon, A.M. and T.J. Ley. 1990. Conservation of the primary structure, organization, and function of the human and mouse β -globin locus-activating regions. *Proc. Natl. Acad. Sci.* **87**: 7693–7697.
- . 1991. Functional properties of the β -globin locus control region in K562 erythroleukemia cells. *Blood* **77**: 2272–2284.
- Ney, P.A., B.P. Sorrentino, K.T. McDonagh, and A.W. Nienhuis. 1990. Tandem AP-1-binding sites within the human β -globin dominant control region functions as an inducible enhancer in erythroid cells. *Genes & Dev.* **4**: 993–1006.
- Nolan, G.P., S. Fiering, J.F. Nicolas, and L.A. Herzenberg. 1988. Fluorescence-activated cell analysis and sorting of viable mammalian cells based on β -D-galactosidase activity after transduction of *Escherichia coli lacZ*. *Proc. Natl. Acad. Sci.* **85**: 2603–2607.
- O'Gorman, S., D.T. Fox, and G.M. Wahl. 1991. Recombinase-mediated gene activation and site-specific integration in mammalian cells. *Science* **251**: 1351–1355.
- Pruzina, S., O. Hanscombe, D. Whyatt, F. Grosveld, and S. Philipson. 1991. Hypersensitive site 4 of the human β -globin locus control region. *Nucleic Acids Res.* **19**: 1413–1419.
- Rudnicki, M.A., P. Schnegelsberg, R.H. Stead, T. Braun, H. Arnold, and R. Jaenisch. 1993. MyoD or Myf-5 is required for the formation of skeletal muscle. *Cell* **75**: 1351–1359.
- Ryan, T.M., R.R. Behringer, N.C. Martin, T.M. Townes, R.D. Palmiter, and R.L. Brinster. 1989. A single erythroid-specific DNase I superhypersensitive site activates high levels of human β -globin gene expression in transgenic mice. *Genes & Dev.* **3**: 314–323.
- Schneeburger, C., P. Speiser, F. Kury, and R. Zeillinger. 1995. Quantitative detection of reverse transcriptase-PCR products by means of a novel and sensitive DNA stain. *PCR Methods Applic.* **4**: 234–238.
- Soriano, P., C. Montgomery, R. Geske, and A. Bradley. 1991. Targeted disruption of the c-src proto-oncogene leads to osteopetrosis in mice. *Cell* **64**: 693–702.
- Sorrentino, B., P. Ney, D. Bodine, and A.W. Nienhuis. 1990. A 46 base pair enhancer sequence within the locus activating region is required for induced expression of the γ -globin gene during erythroid development. *Nucleic Acids Res.* **18**: 2721–2731.
- Talbot, D. and F. Grosveld. 1991. The 5'HS2 of the globin locus control region enhances transcription through the interaction of a multimeric complex binding at two functionally distinct NF-E2 binding sites. *EMBO J.* **10**: 1391–1398.
- Talbot, D., P. Collis, M. Antoniou, M. Vidal, F. Grosveld, and D.R. Greaves. 1989. A dominant control region from the human β -globin locus conferring integrations site independent gene expression. *Nature* **338**: 352–355.
- Talbot, D., S. Philipson, P. Fraser, and F. Grosveld. 1990. Detailed analysis of the site 3 region of the β -globin dominant control region. *EMBO J.* **9**: 2169–2178.
- Taramelli, R., D. Kiuoussis, E. Vanin, K. Bartram, J. Groffen, J. Hurst, and F.G. Grosveld. 1986. $\gamma\beta$ thalassemias 1 and 2 are the result of a 100 kb deletion in the human β -globin cluster. *Nucleic Acids Res.* **14**: 7017–7029.
- Townes, T.M., J. Lingrel, H.Y. Chen, R.L. Brinster, and R.D. Palmiter. 1985. Erythroid-specific expression of human β -globin genes in transgenic mice. *EMBO J.* **4**: 1715–1723.
- Tuan, D., W. Solomon, Q. Li, and I.M. London. 1985. The β -like globin gene domain in human erythroid cells. *Proc. Natl. Acad. Sci.* **82**: 6384–6388.
- Tuan, D., A. Abeliovich, M. Lee-Oldham, and D. Lee. 1987. Identification of regulatory elements of human β -like globin genes. In *Developmental control of globin gene expression* (ed. G. Stamatoyannopoulos and A.W. Nienhuis), pp. 211–220. Alan R. Liss, New York.
- Tuan, D.H., W.B. Solomon, I.M. London, D.P. Lee. 1989. An erythroid-specific, developmental-stage-independent enhancer far upstream of the human “ β -like globin” genes. *Proc. Natl. Acad. Sci.* **86**: 2554–2558.

Targeted deletion of 5'HS2 of the murine beta-globin LCR reveals that it is not essential for proper regulation of the beta-globin locus.

S Fiering, E Epner, K Robinson, et al.

Genes Dev. 1995, **9**:

Access the most recent version at doi:[10.1101/gad.9.18.2203](https://doi.org/10.1101/gad.9.18.2203)

References

This article cites 50 articles, 23 of which can be accessed free at:
<http://genesdev.cshlp.org/content/9/18/2203.full.html#ref-list-1>

License

Email Alerting Service

Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article or [click here](#).