MOLECULAR AND CELLULAR BIOLOGY, June 1996, p. 2906–2912 Vol. 16, No. 6
0270-7306/96/$04.00+0
Copyright © 1996, American Society for Microbiology

Analysis of Mice Containing a Targeted Deletion of β-Globin Locus Control Region 5′ Hypersensitive Site 3

BRUCE A. HUG,[1] ROBIN L. WESSELSCHMIDT,[1] STEVEN FIERING,[2] M. A. BENDER,[3] ELLIOT EPNER,[3] MARK GROUDINE,[3] AND TIMOTHY J. LEY[1]*

Division of Bone Marrow Transplantation and Stem Cell Biology, Departments of Internal Medicine and Genetics, Washington University Medical School, St. Louis, Missouri 63110[1]; Fred Hutchinson Cancer Research Center, Seattle, Washington 98104[2]; and Departments of Radiation Oncology and Medicine, University of Washington School of Medicine, Seattle, Washington 98195[3]

Received 22 January 1996/Returned for modification 7 March 1996/Accepted 13 March 1996

To examine the function of murine β-globin locus control region (LCR) 5′ hypersensitive site 3 (HS3) in its native chromosomal context, we deleted this site from the mouse germ line by using homologous recombination techniques. Previous experiments with human 5′ HS3 in transgenic models suggested that this site independently contains at least 50% of total LCR activity and that it interacts preferentially with the human γ-globin genes in embryonic erythroid cells. However, in this study, we demonstrate that deletion of murine 5′ HS3 reduces expression of the linked embryonic εy- and βH 1-globin genes only minimally in yolk sac-derived erythroid cells and reduces output of the linked adult β (βmajor plus βminor) globin genes by approximately 30% in adult erythrocytes. When the selectable marker *PGK-neo* cassette was left within the HS3 region of the LCR, a much more severe phenotype was observed at all developmental stages, suggesting that *PGK-neo* interferes with LCR activity when it is retained within the LCR. Collectively, these results suggest that murine 5′ HS3 is not required for globin gene switching; importantly, however, it is required for approximately 30% of the total LCR activity associated with adult β-globin gene expression in adult erythrocytes.

The human β-globin gene cluster consists of a set of five functional genes and one pseudogene. The cluster is active exclusively in erythroid cells, and the individual β-like globin genes are expressed during specific developmental stages (33). Sequences local to the individual globin genes are sufficient to direct developmental stage-specific and tissue-specific expression (3, 4, 6, 24, 37, 38), but high-level expression of the globin genes depends on distant regulatory sequences collectively called the locus control region (LCR) (33). The LCR is composed of a series of erythroid cell-specific, developmentally stable DNase I-hypersensitive sites (HSs) (5, 12, 39) that contain the sequences essential for LCR activity (7, 11, 13, 16, 24, 29, 36).

Recent efforts have been directed toward determining the essential sequences within the LCR. Homology comparisons between the LCRs of humans, mice, goats, and rabbits have revealed conservation of the HS cores but not the intervening sequences (17–19, 22, 23, 25, 31). This finding suggests that the conserved sequences define the HS; functional assays have confirmed this hypothesis (12–14, 17, 18, 29, 30, 36). The HSs have been tested individually in transient and stable assays in tissue culture cells and in transgenic mice; 5′ HS2, -3, and -4 do indeed contain the information that directs high-level expression of linked genes.

Recent studies have focused on the activities of the individual HSs within the context of the entire β-globin cluster. These approaches have been undertaken to avoid the complications of studying small transgenes in which distant or undefined regulatory influences may be missing. Transgenic animals have now been made by using large DNA fragments derived from the β-globin cluster (1, 8, 15, 27, 28, 35); although these transgenes are correctly regulated, the levels of expression from genes within the cluster vary with integration sites. These integration site effects may obscure quantitative effects of mutations on globin gene expression. Finally, differences between human and mouse *cis* and *trans* factors and globin gene-switching profiles may affect the interpretations of these transgenic experiments.

As an alternative approach to studying the β-globin LCR, mutations have been made by using homologous recombination and site-specific recombination technology. Kim et al. (20) first modified the human β-globin cluster by using homologous recombination in mouse erythroleukemia cell somatic hybrids containing one copy of human chromosome 11. The Friend virus long terminal repeat driving the hygromycin phosphotransferase gene (*hygro*) was inserted into the human LCR between HS2 and HS1. Even though no LCR sequences were deleted, this insertion inactivated the linked human β-globin gene. This finding suggested that the foreign marker gene was competing with the β-globin gene for the activity of the LCR. Fiering et al. (10) then demonstrated that the β-globin gene could be reactivated by recombinase-mediated deletion of the selectable marker following homologous recombination.

Fiering et al. (9) applied these technologies to delete HS2 in mouse embryonic stem (ES) cells. The functional core of murine HS2 was deleted by homologous recombination, and the selectable marker sequences were then removed by using FLP recombinase. The murine HS2 deletion reduced expression of the adult β-globin genes to approximately 30% of that of the wild-type adult β-globin genes but had only minimal effects on βH 1- and εy-globin gene expression.

The strong LCR activities of human HS3 have caused us to focus our attention on this HS. Several groups have demonstrated that HS3 directs high-level expression of linked human

* Corresponding author. Mailing address: Division of Bone Marrow Transplantation and Stem Cell Biology, Washington University Medical School, Box 8007, 660 South Euclid Ave., St. Louis, MO 63110. Phone: (314) 362-8831. Fax: (314) 362-9333. Electronic mail address: timley@visar.wustl.edu.

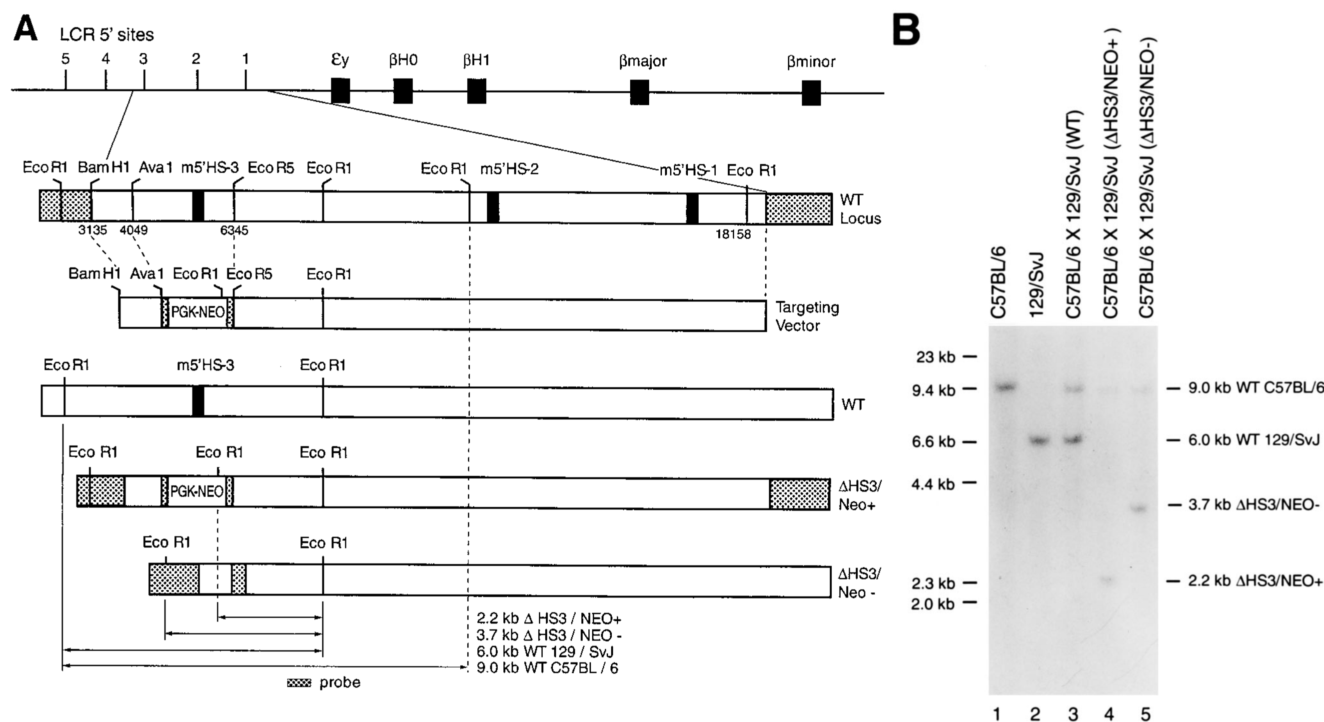

FIG. 1. Strategy for producing 5′ HS3 mutant mice. (A) Maps of wild-type (WT) and mutant 5′ HS3 loci. The top map depicts the wild-type 129/SvJ locus. The positions of murine 5′ HS3 and 5′ HS2 are indicated by the solid black boxes. The unshaded region represents the fragment used to construct the replacement vector, shown immediately below. *loxP* sites flanking *PGK-neo* are represented by hatched boxes. The bottom three maps indicate the positions of *Eco*RI sites in wild-type 129/SvJ, ΔHS3/Neo+, and ΔHS3/Neo− loci. Note the *Eco*RI restriction fragment length polymorphism distinguishing the C57BL/6 and 129/SvJ mouse strains. (B) Southern analysis of *Eco*RI-digested mouse tail DNA. Fragment sizes at the right of the figure correspond to the values below the maps in panel A.

β-globin genes in mouse erythroleukemia cells and transgenic mice and that HS3 is the most active of the four individual LCR sites in assays that require stable integration (13, 18, 29, 30). However, HS3 lacks classical enhancer activity in transient assays (18, 26). Interestingly, HS3 preferentially activates γ- rather than β-globin genes in the erythrocytes derived from the yolk sacs and fetal livers of transgenic mice (14).

To determine the essential activities of murine HS3, we created an HS3 loss-of-function mutation in mice by using ES cells and homologous recombination technology. This mutation has only small effects on εy-globin and βH1-globin gene expression but reduces adult β-globin gene expression in adult animals by approximately 30%. The phenotype of this mutation suggests that HS3 contributes approximately 30% of the total LCR activity required for adult β-globin gene expression and that either it is not required for embryonic globin gene expression or its activity can be rescued by the remaining HS within the LCR.

MATERIALS AND METHODS

Construct design. A positive-selection replacement vector was designed to delete murine HS3 (Fig. 1A). The selectable marker consists of the neomycin phosphotransferase gene (*neo*) driven by the phosphoglycerate kinase 1 gene (*PGK*) promoter (32) and is flanked by 34-bp *loxP* sites to permit CRE recombinase-mediated removal. 5′ (0.7 kb) and 3′ (12.3 kb) targeting sequences were subcloned from a 129/Sv genomic library (Stratagene). After homologous recombination, this vector produces a 2.3-kb *Ava*I-*Eco*RV deletion that includes the entire region containing murine HS3. The vector was linearized with *Sal*I prior to transfection.

ES cell culture. The ES cell line RW4 was derived from 129/SvJ blastocysts. Cells were cultured on feeder layers of murine embryonic fibroblasts in the presence of 1,000 U of leukocyte inhibitory factor per ml (32).

To obtain homologous recombinant ES cell clones, 10 million RW4 cells were transfected with 10 μg of the targeting vector and selected on G418 (0.5 mg/ml [total])-containing medium. Resistant clones were expanded and screened for the deletion of HS3 by Southern analysis.

PGK-neo was deleted from homologous recombinant clone 257 by transfecting cells with a plasmid containing the CRE recombinase plus *PGK-hygro* and selecting on hygromycin B (0.5 mg/ml [total]; Calbiochem). Resistant clones were screened for the deletion of *PGK-neo* by Southern analysis.

Production of mutant mice. C57BL/6J blastocysts were microinjected with 10 to 12 RW4 ES cells containing the desired mutation. Blastocysts were implanted into pseudopregnant Swiss Webster foster mothers to obtain chimeric progeny. Chimeras were mated with C57BL/6J mice, and mutations were transmitted through the germ line to yield F_1 heterozygous animals, which were crossed to produce F_2 animals.

RNA analysis. RNA was prepared as described previously (40). Expression was quantitated by reverse transcription (RT)-PCR, restriction digestion, electrophoresis, and phosphorimaging exactly as described by Fiering et al. (9).

RESULTS

Replacement of murine HS3 with *PGK-neo*. To determine the essential functions of murine HS3 within its native chromosomal context, we deleted HS3 by using homologous recombination techniques in ES cells. Figure 1A displays the targeted region and the replacement vector used create the HS3 mutation. The *PGK-neo* selectable marker is flanked by 0.7 kb of upstream targeting sequence and 12.3 kb of downstream targeting sequence. After homologous recombination, a 2.3-kb deletion that includes all of the known sequences required for murine HS3 function is produced (18). RW4 ES cells from 129/SvJ mice were transfected with the linearized targeting vector, and G418-resistant clones were screened by Southern analysis for replacement of HS3 with *PGK-neo*. Seventy three clones were screened, and two homologous (ΔHS3/Neo+) recombinants were identified.

Homologous recombinant 129/SvJ embryonic stem cells were injected into C57BL/6 blastocysts, and the blastocysts

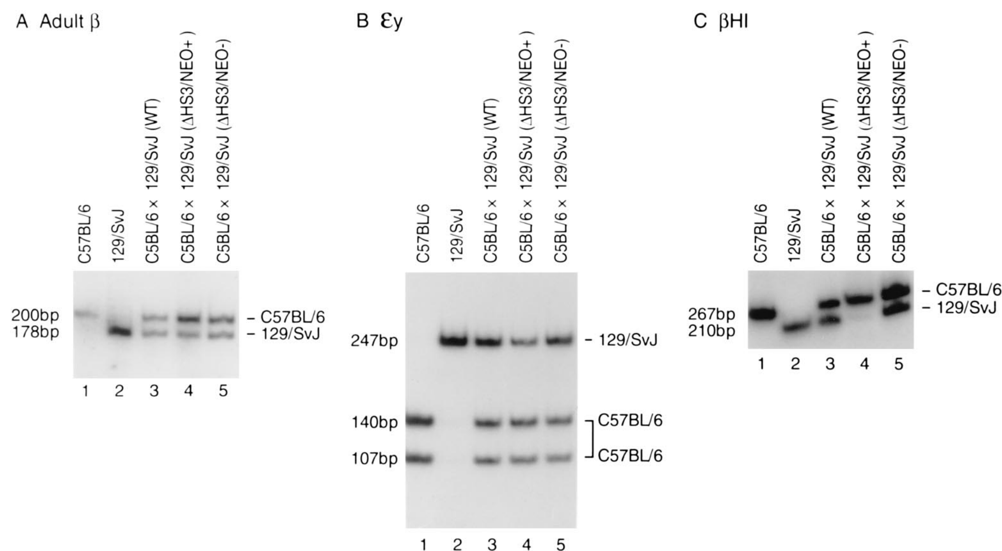

FIG. 2. Representative RT-PCR analysis of globin mRNAs from ΔHS3 mice. (A) Analysis of adult β-globin gene expression in adult blood. Lane 1 shows the *Bst*XI-resistant 200-bp RT-PCR fragment from C57BL/6 blood RNA. Lane 2 shows the 178-bp cleaved RT-PCR product from 129/SvJ blood RNA. Lane 3 displays equivalent levels of product from a wild-type (WT) C57BL/6 × 129/SvJ F1 hybrid. Lane 4 reveals a reduction in the 129/SvJ product relative to C57BL/6 product in blood from a heterozygous mouse carrying the 5′ ΔHS3/Neo+ mutation. Lane 5 reveals a less dramatic reduction in 129/SvJ product relative to C57BL/product in a heterozygous mouse carrying the 5′ ΔHS3/Neo− mutation. (B) Analysis of εy-globin expression in E10.5 RNA. Lane 1 shows the 140- and 107-bp fragments from the cleaved RT-PCR product of C57BL/6 embryonic liver RNA. Lane 2 displays the 247-bp *Xba*I-resistant RT-PCR product from 129/SvJ embryonic liver RNA. Lane 3 shows the products from a wild-type F_1 embryo. The products in lane 4 are from a heterozygous animal carrying the 5′ ΔHS3/Neo+ mutation. Note the reduction in 129/SvJ product relative to C57BL/6 product. The products in lane 5 are from an animal carrying the 5′ ΔHS3/Neo− mutation; the relative reduction in the 129/SvJ product is less severe. (C) Analysis of βH1-globin expression in E10.5 RNA. Lane 1 shows the 267-bp *Xba*I-resistant RT-PCR product from C57BL/6 embryonic liver RNA. Lane 2 shows the 210-bp cleaved product from 129/SvJ RNA. Lane 3 products are from a wild-type F_1 embryo. Lane 4 shows the reduction in 129/SvJ product relative to C57BL/6 product in RNA from a heterozygous embryo carrying the 5′ ΔHS3/Neo+ mutation. Lane 5 products are from a heterozygous embryo carrying the 5′ ΔHS3/Neo− mutation; the relative levels are approximately equivalent.

were then transferred to pseudopregnant females. Two chimeric mice transmitted the mutant 129/SvJ globin cluster through the germ line. Heterozygous animals were identified by Southern analysis as depicted in Fig. 1A. The origin of the 2.2-kb band resulting from *Eco*RI digestion of the ΔHS3/Neo+ homologous recombinant chromosome is shown in the map. Figure 1B, lane 4, shows the Southern analysis of tail DNA derived from a heterozygous ΔHS3/Neo+ mouse (note that C57BL/6 mice lack the polymorphic *Eco*RI site just downstream from HS3). Heterozygous mice carrying this mutation were bred to homozygosity.

Deletion of *PGK-neo* from the site of homologous recombination. We deleted *PGK-neo* from the site of homologous recombination by using the CRE/*lox* site-specific recombinase system. Homologous recombinant ES cells from clone 257 were stably transfected with a plasmid encoding the CRE recombinase and *PGK-hygro*; hygromycin-resistant clones were screened for the deletion of the *PGK-neo* marker by Southern analysis. Six of sixteen clones analyzed contained the deletion of *PGK-neo* (ΔHS3/Neo− [data not shown]).

ΔHS3/Neo− clones were used to make mutant mice as described above. Mice were genotyped by Southern analysis of *Eco*RI-digested tail DNA. Figure 1A reveals the origin of the 3.7-kb band that is generated following the removal of *PGK-neo*; Fig. 1B, lane 5, shows this band on a Southern blot of DNA from a ΔHS3/Neo− heterozygous mouse. Mice containing the ΔHS3/Neo− mutation were also bred to homozygosity.

Quantitation of β-like globin gene expression in mutant mice. Globin gene expression was quantitated in heterozygous mutant animals by using the RT-PCR assay described by Fiering et al. (9). This assay distinguishes between expression from C57BL/6 and 129/SvJ globin clusters by exploiting polymorphisms within the globin genes. The mutant chromosomes are always derived from the embryonic stem cells (129/SvJ). The C57BL/6 chromosome always contains a wild-type β-globin cluster.

The RT-PCR assay measures the effect of HS3 mutations by comparing the expression from the mutant 129/SvJ globin cluster with that from the wild-type C57BL/6 globin cluster. Mixing experiments used in the development of the assay proved that it performs accurately over ratios from 9:1 to 1:9 (9). Additionally, for every experiment performed in this study, we confirmed the accuracy of the RT-PCR assay by using wild-type controls and mixtures of C57BL/6 and 129/SvJ RNA in 1:5 and 5:1 ratios (data not shown).

The effect of the HS3 mutations on adult β (βmajor plus βminor)-globin expression was first examined by RT-PCR. RNA derived from livers of day 14.5 embryos or the blood of adult animals was analyzed. Primers were designed to span intron 2 of both adult β-globin genes and amplify RNA from both 129/SvJ and C57BL/6 chromosomes. Restriction digestion of the RT-PCR products with *Bst*XI cleaves the 129/SvJ product, but not the C57BL/6 product, as a result of a polymorphism between the two mouse strains. The digested samples were electrophoresed on 6% polyacrylamide gels. Figure 2A shows representative results of this assay.

Phosphorimaging was used to quantify the relative reductions in adult β-globin expression produced by the ΔHS3/Neo+ and ΔHS3/Neo− mutations (Fig. 2A and 3). Analysis of blood from a wild-type F_1 hybrid animal demonstrated that levels of expression from the two chromosomes are virtually equivalent. In contrast, the ΔHS3/Neo+ heterozygotes express adult β-globin from the mutant 129/SvJ chromosome at 44% ± 4% of the level of the adult β genes from the wild-type C57BL/6 chromosome. The ΔHS3/Neo− mutation reduces adult globin gene expression to 70% ± 3% of the wild-type

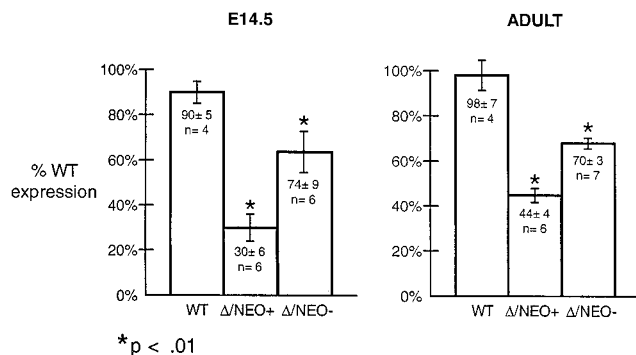

FIG. 3. Adult β-globin gene expression in embryonic livers at day 14.5 and in adult blood. RNA was analyzed by RT-PCR as described for Fig. 2 and quantified by phosphorimaging. The level of adult β-globin gene expression from the 129/SvJ chromosome was recorded as a percentage of expression from the adult β-globin genes of the wild-type (WT) C57BL/6 chromosome.

level. Similarly, at embryonic day 14.5 (E14.5), total liver RNA from a wild-type control shows that levels of adult β-globin expression from both chromosomes are virtually identical. Levels of adult β-globin expression from the ΔHS3/Neo+ and ΔHS3/Neo− mutant clusters, however, are 30% ± 6% and 74% ± 9%, respectively, of the wild-type level. These results show that deletion of HS3 reduces expression of the adult β-globin genes by 30% and that the presence of *PGK-neo* within the HS3 region of the LCR reduces expression by an additional 25 to 40%.

Because we removed *PGK-neo* by stably transfecting ES cells with the CRE recombinase vector, it was necessary to confirm that the recombinase gene did not influence globin gene expression in mice. The recombinase segregates independently of the β-globin cluster, and so only half of the progeny of chimeric animals contain the CRE gene. Southern analysis was used to determine which animals contained the recombinase and which did not (data not shown). We found no difference in globin expression of animals with and without the CRE gene and therefore conclude that the presence of the CRE gene does not affect globin gene expression.

The effects of the HS3 mutations on embryonic globin gene expression was next determined. RNA was prepared from whole embryos at E9.5 and E10.5. RT-PCR was performed as described above, using primers specific for the βH1- and εy-globin genes. βH1 RT-PCR products from the 129/SvJ chromosome are susceptible to *Xba*I cleavage, while βH1 products from the C57BL/6 chromosome are resistant. The converse is true for εy-globin RT-PCR products: 129/SvJ products are resistant, while C57BL/6 products are cleaved by *Xba*I. Figures 2B and C demonstrate the analysis of εy- and βH1-globin RT-PCR products, respectively.

εy-globin expression is affected only minimally by the HS3 deletion (Fig. 2B and 4). The ΔHS3/Neo+ mutation reduces εy-globin expression to 28% ± 6% of the wild-type level on E9.5 and to 42% ± 4% on E10.5. The ΔHS3/Neo− mutation reduces εy-globin expression to 77% ± 7% of the wild-type level on E9.5 and 83% ± 9% on E10.5. Although the absolute reductions are relatively small, these differences are both significant at $P \leq 0.01$.

βH1-globin expression is reduced by both HS3 mutations, as shown in Fig. 2C and 5. At E9.5 and E10.5, levels of βH1-globin expression from the ΔHS3/Neo+ mutant cluster are 30% ± 5% and 17% ± 5% of that of the wild-type βH1-globin gene. However, levels of βH1-globin expression from the ΔHS3/Neo− mutant are 81% ± 14% of the wild-type level on E9.5 and 76% ± 13% on E10.5. This result indicates that HS3 has only a small impact on βH1-globin expression before the embryonic-to-adult switch. However, *PGK-neo* substantially reduces the expression of the βH1-globin gene if it is left in the HS3 position of the LCR.

Neither the ΔHS3/Neo+ nor the ΔHS3/Neo− mutation prevents the developmental switch from embryonic expression to adult expression. RT-PCR was performed on wild-type and mutant RNAs from day 9.5, 10.5, and 14.5 embryos and from adult blood. Adult β-globin mRNA was not detected at days 9.5 and 10.5. εy- and βH1-globin mRNAs were not detected in adult blood (data not shown). The HS3 mutations appear to influence only the level of expression from the β-globin cluster, not the pattern of expression during development.

Partial embryonic lethality and mild adult thalassemia in mice homozygous for the ΔHS3/Neo+ mutation. While homozygous animals bearing the ΔHS3/Neo+ mutation are viable and not runted, they are born at non-Mendelian frequencies. Ninety offspring from heterozygous ΔHS3/Neo+ matings were obtained. The distribution of wild-type to heterozygous to homozygous animals was 26:52:12. This finding suggests that at some time during development (perhaps E9.5 to E10.5), the level of β-like globin chains in homozygotes approaches the minimum necessary for viability, and so only about half of the embryos are able to survive this stage.

Table 1 shows the data from the complete blood counts of wild-type F_1 hybrids and from heterozygous and homozygous ΔHS3/Neo+ mice. Homozygous animals bearing the ΔHS3/

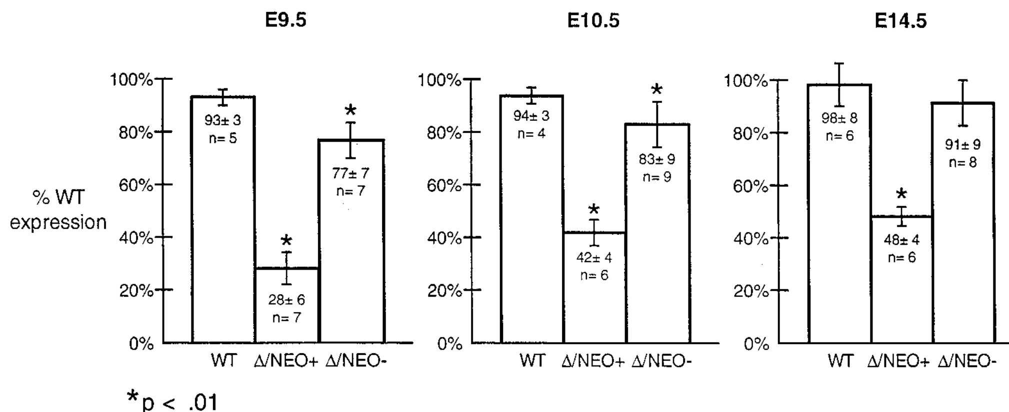

FIG. 4. εy-globin gene expression at E9.5, E10.5, and E14.5. Results are displayed as described for Fig. 3.

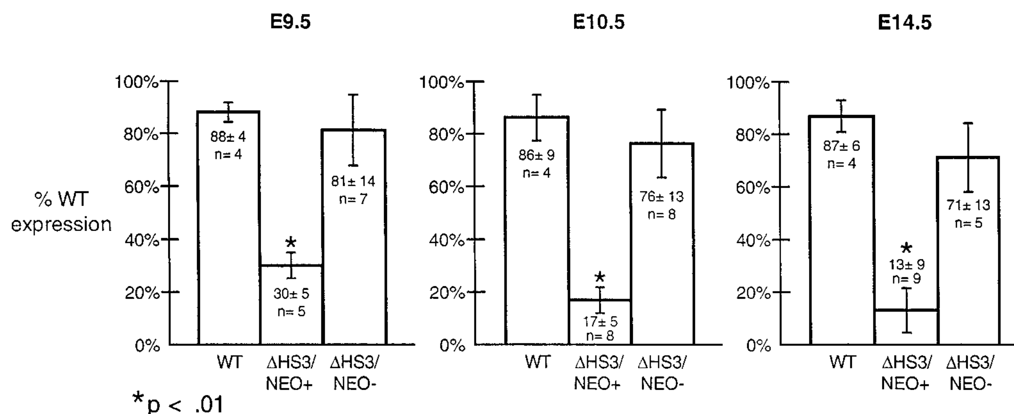

FIG. 5. βH1-globin gene expression at E9.5, E10.5, and E14.5. Results are displayed as described for Fig. 3.

Neo+ mutation have reduced mean cell volumes, hematocrits, and mean cell hemoglobin concentrations; reticulocyte counts are significantly elevated in these animals. These hematologic values are consistent with the mild thalassemia syndrome that would be predicted from a ~55% reduction in the total adult β-globin gene output.

DISCUSSION

The goal of this study was to determine the essential functions of murine β-globin 5′ HS3 within its native chromosomal context. We therefore deleted HS3 by homologous recombination and then removed the selectable marker gene from the mutant LCR by site-specific recombination. Surprisingly, expression from the linked β-like globin genes was reduced only slightly by this mutation.

Our HS3 deletion removed all of the murine HS3 sequences known to be necessary for HS3 function. The mutation removed the murine sequences homologous to the conserved HS3 regions from humans, goats, and rabbits (17, 22, 23, 29, 30); since all sequences with a high degree of conservation were removed, our deletion was likely to be extensive enough to eliminate all sequences important for HS3 activity. Importantly, our deletion includes the 1.9-kb *Hin*dIII fragment shown previously to contain the functional murine HS3 core (18). The murine sequences immediately flanking this region lack LCR activities in standard in vitro assays (18). Therefore, even if nonconserved elements contributing to HS3 activity remain within our mutants, these elements contain no residual activities when separated from the HS3 core.

A series of experiments with human HS3 suggested that it contained powerful, independent LCR activities. Fraser et al. (13) showed that when individual HSs were linked to the human β-globin gene and tested in transgenic mice, the sum of the expression conferred by the sites tested individually was greater than the level for the intact LCR. In fact, the expression conferred by HS3 alone was 70% of the total LCR activity. However, when a transgene was linked to a ΔHS3 LCR, expression was 50% of the wild-type level. These data implied that the activities of some HSs are independent but that some may be redundant. Later, Grosveld and colleagues (14, 35) described the phenotype of mice expressing cosmid transgenes that included the human γ- and β-globin genes. When the HSs were tested individually (14), the sum of the activities approximated that of a cluster linked to an intact LCR (35). The levels of human globin gene expression in these studies suggested that the HS activities would be additive. In more recent experiments, Ellis et al. (7a) demonstrated that HS3, but not HS2, was capable of directing β-globin gene expression from several independent integration sites in transgenic mice containing single-copy transgenes. On the basis of these results and accompanying chromatin analyses, the authors proposed that HS3 contained the dominant chromatin-opening activity of the LCR.

We found that the deletion of HS3 reduced expression of the individual β-like globin genes from 10 to 30%. Similar results were obtained when Fiering et al. (9) deleted murine HS2 by using homologous recombination approaches identical to these described here (Table 2). The minimal alterations in globin gene expression obtained upon deletion of HS2 or HS3 suggest that neither of these HSs contains the dominant chromatin-opening activity of the locus. Although HS2 and HS3 have high levels of LCR activity in transgenic mouse assays, the effects of their removal on murine globin gene expression are small but significant, especially for the adult β-globin genes. While conclusions about the redundancy of HSs cannot be made until the remaining sites have been deleted, it appears that the loss of a single HS can largely be compensated for by residual LCR activity in the remaining HSs. However, since HS2 and HS3 deletions both cause a 30% reduction in adult β-globin expres-

TABLE 1. Complete blood counts of ΔHS3/Neo+ mice

Genotype	*n*	Blood counts (mean ± SD)					
		Hemoglobin (g/dl)	Hematocrit (%)	Mean red cell vol (fl)	% Reticulocytes	Leukocytes (10^3/mm^3)	Platelets (10^3/mm^3)
+/+	4	14.8 ± 0.28	44.4 ± 0.21	46.4 ± 1.3	2.4 ± 1.3	8.6 ± 2.6	709 ± 254
+/−	6	16.2 ± 1.5	48.1 ± 4.6	45.2 ± 1.4	4.0 ± 3.0	8.9 ± 2.9	929 ± 167
−/−	5	13.8 ± 0.33	38.8 ± 2.2[a]	38.8 ± 2.2[a]	12.7 ± 3.2[a]	12.2 ± 4.3	1,022 ± 228

[a] $P < 0.01$.

TABLE 2. Phenotypes of ΔHS2 and ΔHS3 mutations

Mutation	Globin expression (Δ/Neo−, % of wt)			Embryonic viability (Δ/Neo+, %)	Neo+ reduction of globin gene expression (%)
	Adult β	εy	βH1		
ΔHS2	70	100	85	0	40–90
ΔHS3	70	90	90	50	25–60

sion and small reductions in βH1- and εy-globin expression, our results suggest that neither site can fully compensate for the absence of the other.

Examination of β-globin cluster transgenes in association with partial LCRs has suggested that individual human LCR HS may preferentially interact with individual globin genes. Fraser et al. (14) linked a cosmid containing the human γ- and β-globin genes to individual HSs. While HS2 interacted equally with γ- and β-globin genes, HS3 preferentially increased γ-globin expression, and HS4 preferentially increased β-globin expression. Additionally, Peterson et al. (28) described deletions of individual HSs in the context of a yeast artificial chromosome that contained the entire β-globin locus. Deletion of HS2 uniformly reduced expression from the cluster, but deletion of HS3 preferentially reduced expression of the ε-globin gene. These studies implied that individual HSs may function preferentially either with specific genes or at specific stages.

The deletion of murine HS2 by homologous recombination, however, does not produce the phenotype predicted by the transgenic mouse experiments described above. While HS2 seems to interact with embryonic and adult globin genes equally in tissue culture cells and in transgenic mice, the deletion in the mouse β-globin cluster reduces only adult β-globin expression significantly.

In this study, as in the study of HS2, the deletion of HS3 does not display the preferential interactions predicted by the transgenic studies (Table 2). Since εy- and βH1-globin expression are reduced only mildly by the HS3 deletion, HS3 must not contain embryonic stage-specific activities; however, the preferential reduction of adult β-globin gene expression suggest that HS3 may contain some adult-specific activity. Previous studies using tissue culture cells in which HS3 was inactive in the embryonic erythroid K562 environment but active in the adult erythroid environment of mouse erythroleukemia cells support these observations (18). Because we detected appropriately regulated globin gene expression from the mutant cluster, HS3 is not required for globin switching; this result was predicted, however, since human globin cluster transgenes switch appropriately in the absence of the LCR (34).

The mild phenotype of the ΔHS3/Neo− mice contrasts with the severe effect observed when human HS3 was deleted from a yeast artificial chromosome containing the human β-globin cluster. Bungert et al. (2) deleted human HS3 from a 150-kb fragment containing the human β-globin cluster and found that globin expression was completely eliminated at each stage of development in transgenic mice. The severity of reduction (>90%) in two independent founder lines makes this finding unlikely to have resulted from integration site-specific effects. Interestingly, the HS3 mutation in that study removes only 225 bp containing the HS3 core sequence. In contrast, the mutation of murine HS3 reported here removes a 2.3-kb fragment, which includes the core and flanking sequences. Bungert et al. (2) hypothesized that the mutant yeast artificial chromosome may contain residual HS3 motifs outside the core that lead to a dominant negative phenotype. If the mutant human HS3 lacked its transcriptional activating property but could still interact with the other HSs or the β-like globin genes, it could inhibit the activity of the entire LCR. In contrast, the more extensive deletion of murine HS3 may eliminate both transcriptional activation and interactions with other regions of the β-globin gene cluster. Consequently, the redundant murine HS activities could compensate for the loss of HS3 function. This explanation suggests that the large deletion of murine HS3 may more accurately represent the true loss-of-function phenotype.

This study confirms the importance of deleting *PGK-neo* from the site of homologous recombination prior to analyzing the phenotypes of LCR mutants. The insertion of *PGK-neo* reduced expression from the β-globin genes of the mutant cluster as much as the deletion of HS3 itself. The insertion of *PGK-neo* into the HS3 region provides the first example of a disruptive effect when a selectable marker is placed upstream of HS2. Previously, markers had been interposed only between the active HSs (HS2, -3, and -4) and the globin gene cluster. In this experiment, however, HS2 lies between *PGK-neo* and the cluster, but the presence of *PGK-neo* still reduces globin expression from 25 to 60%. This reduction is perhaps less severe than the 40 to 90% reductions observed when *PGK-neo* replaced HS2.

The mechanism by which *PGK-neo* disrupts the LCR is still unclear. One possibility is that transcription through the LCR can disrupt the activity of the HSs downstream from *PGK-neo*. For the ΔHS2/Neo+ mutation, *PGK-neo* is transcribed 5′ to 3′ with respect to the β-globin cluster; for ΔHS3/Neo+, *PGK-neo* is transcribed 3′ to 5′. If ΔHS2/Neo+ transcription disrupted HS1 function, we would expect little or no effect, since HS1 has minimal function (13, 21, 26). However, if ΔHS3/Neo+ transcription disrupted HS4 function, an additional active HS would be disabled and the resulting defect in the LCR activity should be more severe. Since the ΔHS2/Neo+ phenotype is more severe than the ΔHS3/Neo+ phenotype (Table 2), we consider this mechanism to be unlikely. Changes in spacing between HSs probably do not affect LCR function, since naturally occurring polymorphisms that change the spacing of sites without affecting globin gene expression have been described (19). Finally, it has been suggested that *PGK-neo* competes with the globin genes for an interaction with the LCR (9, 10, 20). This hypothesis is supported by our finding that when *PGK-neo* replaces HS3 and is upstream from HS2, the impact of the insertion upon globin gene expression is less severe than when it replaces HS2 (9). Presumably, the globin cluster can compete more effectively for HS2 activity when *PGK-neo* is placed in an upstream position. The mechanism of *PGK-neo*'s effects will be more clear after additional insertion positions are examined within the LCR.

The viabilities of homozygous ΔHS2/Neo+ mice and homozygous ΔHS3/Neo+ mice differ. Homozygous ΔHS2/Neo+ mutant mice die in utero at approximately days 10.5 to 11.5. In contrast, some ΔHS3/Neo+ mice survive, but they are born at half of the expected Mendelian frequency. Embryonic globin expression is not reduced as severely by the ΔHS3/Neo+ mutation as by the ΔHS2/Neo+ mutation: the ΔHS3/Neo+ mutation reduced εy-globin expression to 28% and βH1-globin expression to 30% of the wild-type level on E9.5, but the ΔHS2/Neo+ mutation reduced εy-globin expression to 11% and βH1-globin expression to 43% of the wild-type level on E9.5. Since εy-globin contributes a greater percentage of total embryonic globin than βH1-globin at E9.5 (35, 41), the effect on survival may largely be due to this defect. From these data, it seems likely that the ΔHS3/Neo+ mutation reduces εy-globin gene expression to the threshold necessary for survival.

In conclusion, the deletion of murine HS3 from the β-globin LCR reveals a phenotype that is remarkably similar to that observed in mice deficient for HS2 (Table 2). Neither of these sites is essential for embryonic globin gene expression, but both of them contribute approximately one-third of the total LCR activity for adult β-like globin gene expression. These data imply that each of these HSs has some activity that is not duplicated by other sites within the LCR. Finally, these experiments have clearly shown that murine HS3 does not preferentially interact with embryonic globin genes, as was predicted from previous experiments using large DNA fragments in transgenic mice. Although the explanation for this difference is not yet clear, future experiments designed to resolve these differences may reveal important new insights regarding LCR biology.

ACKNOWLEDGMENTS

This work was supported by NIH grants DK 38682 (T.J.L.) and HL48356 and DK44746 (M.G.).

We thank Pam Goda for excellent animal care, Agnes Telling for technical assistance, and Nancy Reidelberger for preparation of the manuscript.

REFERENCES

1. **Behringer, R. R., T. M. Ryan, R. D. Palmiter, R. L. Brinster, and T. M. Townes.** 1990. Human γ- to β-globin gene switching in transgenic mice. Genes Dev. **4:**380–389.
2. **Bungert, J., U. Dave, K. C. Lim, K. Lieuw, J. Shavit, Q. Liu, and J. Engel.** 1995. Synergistic regulation of human β-globin gene switching by locus control region elements HS3 and HS4. Genes Dev. **9:**3083–3096.
3. **Chada, K., J. Magram, and F. Costantini.** 1986. An embryonic pattern of expression of a human fetal globin gene in transgenic mice. Nature (London) **319:**685–688.
4. **Chada, K., J. Magram, K. Raphael, G. Radice, E. Lacy, and F. Costantini.** 1985. Specific expression of a foreign β-globin gene in erythroid cells of transgenic mice. Nature (London) **314:**377–380.
5. **Dhar, V., A. Nandi, C. L. Schildkraut, and A. Skoultchi.** 1990. Erythroid-specific nuclease-hypersensitive sites flanking the human β-globin domain. Mol. Cell. Biol. **10:**4324–4333.
6. **Dillon, N., and F. Grosveld.** 1991. Human γ-globin genes silenced independently of other genes in the β-globin locus. Nature (London) **350:**252–254.
7. **Driscoll, M., C. Dobkin, and B. Alter.** 1989. γδβ-Thalassemia due to a *de novo* mutation deleting the 5′ β-globin gene activation-region hypersensitive sites. Proc. Natl. Acad. Sci. USA **86:**7470–7474.

7a. **Ellis, J., K. C. Tan-Un, A. Harper, D. Michalovich, N. Yannoutsos, S. Philipsen, and F. Grosveld.** 1996. A dominant chromatin opening activity in 5′ hypersensitive site 3 of the human β-globin locus control region. EMBO J. **15:**562–568.

8. **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 (London) **344:**309–313.
9. **Fiering, S., E. Epner, K. Robinson, Y. Zhuang, A. Telling, M. Hu, D. I. K. Martin, T. Enver, T. J. Ley, and M. Groudine.** 1995. Targeted deletion of 5′ HS-2 of the murine β-globin LCR reveals that it is not essential for proper regulation of the β-globin locus. Genes Dev. **9:**2203–2213.
10. **Fiering, S., C. Kim, E. Epner, and M. 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. USA **90:**8469–8473.
11. **Forrester, W., U. Novak, R. Gelinas, and M. Groudine.** 1989. Molecular analysis of the human β-globin locus activation region. Proc. Natl. Acad. Sci. USA **86:**5439–5443.
12. **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. USA **83:**1359–1363.
13. **Fraser, P., J. Hurst, P. Collis, and F. Grosveld.** 1990. DNase1 hypersensitive sites 1, 2, and 3 of the human β-globin dominant control region direct position-independent expression. Nucleic Acids Res. **18:**3503–3507.
14. **Fraser, P., S. Pruzina, M. Antoniou, and F. Grosveld.** 1993. Each hypersensitive site of the human β-globin locus control region confers a different developmental pattern of expression on the globin genes. Genes Dev. **7:**106–113.
15. **Gaensler, K., M. Kitamura, and Y. Kan.** 1993. Germ-line transmission and developmental regulation of a 150-kb yeast artificial chromosome containing the human β-globin locus in transgenic mice. Proc. Natl. Acad. Sci. USA **90:**11381–11385.
16. **Grosveld, F., G. 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.
17. **Hardison, R., J. Xu, J. Jackson, J. Mansberger, O. Selifonova, B. Grotch, J. Biesecker, H. Petryowska, and W. Miller.** 1993. Comparative analysis of the locus control region of the rabbit β-like globin gene cluster: HS3 increases transient expression of an embryonic ε-globin gene. Nucleic Acids Res. **21:**1265–1272.
18. **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.
19. **Jimenez, G., K. B. Gale, and T. Enver.** 1992. The mouse β-globin locus control region: hypersensitive sites 3 and 4. Nucleic Acids Res. **20:**5797–5803.
20. **Kim, C., E. Epner, W. 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.
21. **Kulozik, A. E., S. Bail, A. Bellan-Koch, C. R. Bartram, E. Kohne, and E. Kleihauer.** 1991. The proximal element of the β globin locus control region is not functionally required in vivo. J. Clin. Invest. **87:**2142–2146.
22. **Li, Q., B. Zhou, P. Powers, T. Enver, and G. Stamatoyannopoulos.** 1990. β-Globin locus activation regions: conservation of organization, structure, and function. Proc. Natl. Acad. Sci. USA **87:**8207–8211.
23. **Li, Q., B. Zhou, P. Powers, T. Enver, and G. Stamatoyannopoulos.** 1991. Primary structure of the goat β-globin locus control region. Genomics **9:**488–499.
24. **Magram, J., K. Chada, and F. Costantini.** 1985. Development regulation of a cloned adult β-globin gene in transgenic mice. Nature (London) **315:**338–340.
25. **Moon, A., 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. USA **87:**7693–7697.
26. **Moon, A., and T. J. Ley.** 1991. Functional properties of the β-globin locus control region in K562 erythroleukemia cells. Blood **77:**2272–2284.
27. **Peterson, K., C. Clegg, C. Huxley, B. Josephson, H. Haugen, T. Furukawa, and G. Stamatoyannopoulos.** 1993. Transgenic mice containing a 248-kb yeast artificial chromosome carrying the human β-globin locus display proper developmental control of human globin genes. Proc. Natl. Acad. Sci. USA **90:**7593–7597.
28. **Peterson, K. R., P. A. Navas, C. H. Clegg, and G. Stamatoyannopoulos.** 1995. Use of β-YAC mice for studying LCR function: effect of 5′ HS-2 or HS-3 deletions on globin gene expression during development. Blood **86:**586a.
29. **Philipsen, S., S. Pruzina, and F. Grosveld.** 1993. The minimal requirements for activity in transgenic mice of hypersensitive site 3 of the β globin locus control region. EMBO J. **12:**1077–1085.
30. **Philipsen, S., D. Talbot, P. Fraser, and F. Grosveld.** 1990. The β-globin dominant control region: hypersensitive site 2. EMBO J. **9:**2159–2167.
31. **Shehee, W., D. Loeb, N. Adey, F. Burton, N. Casavant, P. Cole, C. Davies, R. McGraw, S. Schichman, D. Severynse, C. Voliva, F. Weyter, G. Wisely, M. Edgell, and C. Hutchison III.** 1989. Nucleotide sequence of the BALB/c mouse β-globin complex. J. Mol. Biol. **205:**41–62.
32. **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:**639–702.
33. **Stamatoyannopoulos, G., A. W. Nienhuis, P. Leder, P. W. Majerus (ed.).** 1994. Hemoglobin switching, p. 107–155. *In* G. Stamatoyannopoulos and A. W. Nienhuis (ed.), The molecular basis of blood diseases. W. B. Saunders, Philadelphia.
34. **Starck, J., R. Sarkar, M. Romana, A. Bhargava, A. Scarpa, M. Tanaka, J. Chamberlain, S. Weissman, and B. Forget.** 1994. Developmental regulation of human γ- and β-globin genes in the absence of the locus control region. Blood **84:**1656–1665.
35. **Strouboulis, J., N. Dillon, and F. Grosveld.** 1992. Developmental regulation of a complete 70-kb human β-globin locus in transgenic mice. Genes Dev. **6:**1857–1864.
36. **Talbot, D., P. Collis, M. Antoniou, M. Vidal, F. Grosveld, and D. Greaves.** 1989. A dominant control region from the human β-globin locus conferring integration site-independent gene expression. Nature (London) **338:**352–355.
37. **Townes, T., J. Lingrel, H. Chen, R. Brinster, R. Palmiter.** 1985. Erythroid-specific expression of human β-globin genes in transgenic mice. EMBO J. **4:**1715–1723.
38. **Trudel, M., J. Magram, L. Bruckner, and F. Costantini.** 1987. Upstream Gγ-globin and downstream β-globin sequences required for stage-specific expression in transgenic mice. Mol. Cell. Biol. **7:**4024–4029.
39. **Tuan, D., W. Solomon, Q. Li, and I. London.** 1985. The "β-like globin" gene domain in human erythroid cells. Proc. Natl. Acad. Sci. USA **82:**6384–6388.
40. **Ulrich, M., and T. J. Ley.** The function of normal and mutated γ-globin gene promoters in electroporated K562 erythroleukemia cells. 1990. Blood **75:** 990–999.
41. **Whitelaw, E., S. Tsai, P. Hogben, and S. Orkin.** 1990. Regulated expression of globin chains and the erythroid transcription factor GATA-1 during erythropoiesis in the developing mouse. Mol. Cell. Biol. **10:**6596–6606.