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The human *β-globin* locus control region confers an early embryonic erythroid-specific expression pattern to a basic promoter driving the bacterial *lacZ* gene

Rita Tewari[1], Nynke Gillemans[1], Alex Harper[2], Mark Wijgerde[1], Gaetano Zafarana[1], Dubravka Drabek[1], Frank Grosveld[1,*] and Sjaak Philipsen[1]

[1]Erasmus University Rotterdam, Department of Cell Biology, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands
[2]Transgenic Research Institute, The Rayne Institute, St Thomas' Hospital, London SE1 7EH, UK

*Author for correspondence (e-mail: vanderkamp@ch1.fgg.eur.nl)

SUMMARY

The *β-globin* locus control region (LCR) is contained on a 20 kb DNA fragment and is characterized by the presence of five DNaseI hypersensitive sites in erythroid cells, termed 5′HS1-5. A fully active 6.5 kb version of the LCR, called the μLCR, has been described. Expression of the β-like globin genes is absolutely dependent on the presence of the LCR. The developmental expression pattern of the genes in the cluster is achieved through competition of the promoters for the activating function of the LCR. Transgenic mice experiments suggest that subtle changes in the transcription factor environment lead to the successive silencing of the embryonic ε-globin and fetal γ-globin promoters, resulting in the almost exclusive transcription of the *β-globin* gene in adult erythropoiesis. In this paper, we have asked the question whether the LCR and its individual hypersensitive sites 5′HS1-4 can activate a basic promoter in the absence of any other globin sequences. We have employed a minimal promoter derived from the mouse *Hsp68* gene driving the bacterial *β-galactosidase (lacZ)* gene. The results show that the μLCR and 5′HS3 direct erythroid-specific, embryonic expression of this construct, while 5′HS1, 5′HS2 and 5′HS4 are inactive at any stage of development. Expression of the μLCR and 5′HS3 transgenes is repressed during fetal stages of development. The transgenes are in an inactive chromatin conformation and the *lacZ* gene is not transcribed, as shown by in situ hybridization. These data are compatible with the hypothesis that the LCR requires the presence of an active promoter to adopt an open chromatin conformation and with models proposing progressive heterochromatization during embryogenesis. The results suggest that the presence of a *β-globin* gene is required for LCR function as conditions become more stringent during development.

Key words: locus control region, β-globin, *lacZ* gene, basic promoter, erythropoiesis, embryonic expression pattern, DNaseI hypersensitive sites, transgenic mice, mouse

INTRODUCTION

The locus control region (LCR) of the human *β-globin* multigene cluster is a cis-acting regulatory element present 5-25 kb upstream of the *ε-globin* gene. The cluster contains five developmentally regulated genes in the order 5′-embryonic (ε), fetal (γ^G and γ^A) and adult (δ and β)-3′. The regulatory function of the LCR became first apparent from the study of a Dutch thalassemia (Kioussis et al., 1983) and other naturally occurring deletions of the region where the intact globin gene is not activated (Driscoll et al., 1989; Taramelli et al., 1986). It has been shown that deletion of the LCR not only affects expression of the genes, but also the chromatin structure of the locus over a distance of at least 200 kb (Forrester et al., 1990).

Chromatin analysis of the LCR showed that it contains a set of developmentally stable DNaseI-hypersensitive sites (HS) termed 5′HS1-5 (Grosveld et al., 1987; Tuan et al., 1985). Various studies have been undertaken to understand the nature and function of this region. These studies revealed that the LCR, when coupled to globin genes in cis, confers high level, copy-number dependent- and position-independent expression to these genes in erythroid tissues in transgenic mice (Grosveld et al., 1987; Ryan et al., 1989; Talbot et al., 1989). 5′HS2-4 also have these properties when linked as single HS to the *β-globin* gene (Fraser et al., 1990). Transfection studies stated that only 5′HS2 acts as an enhancer in transient assays (Tuan et al., 1989; Moon and Ley, 1991).

A fully active 6.5 kb version of the LCR containing 5′HS1-4, termed the μLCR, has been described (Talbot et al., 1989). Further dissection of the μLCR revealed that the activity of each HS can be localized to 200-300 bp core fragments coinciding with the position of hypersensitivity in chromatin (Philipsen et al., 1990; Pruzina et al., 1991; Talbot et al., 1990). The minimal element required for LCR activity of 5′HS3 was assigned to a 125 bp fragment (Philipsen et al., 1993). Interestingly, recent observations from our laboratory suggest that 5′HS3 is the only site that possesses significant chromatin

opening activity in single copy transgenic mice (Ellis et al., 1996).

The LCR core fragments all contain binding sites for the erythroid-specific protein GATA-1, and 'GGTGG' or 'CACC' box motifs binding members of the Sp1 family of transcription factors (Ellis et al., 1993; Philipsen et al., 1990, 1993; Pruzina et al., 1991, 1994; Talbot et al., 1990). This combination of binding sites is also found in the promoters and enhancers of the *β-globin* locus as well as many other erythroid-specific genes (Philipsen et al., 1990 and references therein). Hence, we have asked whether the μLCR and its individual HS can function in the absence of any other erythroid regulatory elements. To answer this, we have used the bacterial *lacZ* gene driven by a non-erythroid, 80 bp minimal promoter derived from the mouse *heat shock protein 68 (Hsp68)* gene (Kothary et al., 1987, 1988, 1989). The results show that only the μLCR and 5′HS3 are able to direct the expression of this gene in the erythroid lineage reproducibly. Interestingly, expression is limited to the embryonic stages of development, after which the chromatin of the transgenes is in an inactive conformation and transcription is repressed. Our data suggest that the presence of a *β-globin* gene is essential for LCR function in definitive erythroid cells, implying that there are more stringent requirements for erythroid-specific gene expression in later developmental stages than during the embryonic period.

MATERIALS AND METHODS

Transgenic mice and embryo collection

Five different fragments, 5′HS4 (2.1 kb *Bam*HI-*Xba*I), 5′HS3 (1.9 kb *Hin*dIII), 5′HS2 (1.5 kb Asp718-*Bgl*II), 5′HS1(1 kb *Sst*I (partial)-*Hin*d III) and μLCR (6.5 kb, containing 5′HS1-4) (Talbot et al., 1989) were linked to the mouse *Hsp68* minimal promoter driving the bacterial *lacZ* reporter gene. Each of these fragments was isolated as described (Kollias et al., 1986). The fragments were dissolved in micro-injection buffer (10 mM Tris-Cl, pH 7.5 and 0.1 mM EDTA) at a concentration of 1-2 μg/ml and microinjected into the pronucleus of FVB fertilized mouse eggs. The injected eggs were transferred into the oviducts of pseudo-pregnant foster females as described (Kollias et al., 1986).

For the 'transient' assay, the foster females were killed 13.5 days after egg transfer and the fetuses were collected from the uterus. For stable lines, transgenic founders were bred to non-transgenic mice. After the lines were established the F1 or F2 males were used for mating with non-transgenic females for the collection of embryos and fetuses at various stages of development.

DNA analysis

Genomic mouse DNA was prepared from part of the body of the fetuses or tail clips from adult mice. The DNA was cut with *Eco*RI, Southern blotted and probed with *lacZ* to detect the transgene. A fragment from the mouse *ThyI* gene was used as a loading control (Talbot et al., 1990).

DNaseI hypersensitive site mapping

Nuclei were isolated from 8-10 transgenic fetal livers (embryonic day 13.5, E13.5) and DNaseI hypersensitive site mapping was performed as described (Ellis et al., 1996). After this treatment the DNA was isolated and analyzed by digesting with *Eco*RI. The blots were first hybridized with the *Sau*3AI-*Xba*I fragment, coordinates 39819-40269 (Shehee et al., 1989) detecting the hypersensitive site at the promoter of the mouse *β-major* gene, and then with the *lacZ* probe to detect hypersensitive sites at the transgene.

LacZ expression in transgenic embryos and fetuses

For the collection of mice at various developmental stage, transgenic males from each line were kept with wild-type FVB female mice. The day of the vaginal plug was taken as E0.5. The embryos were dissected out of the decidua and fixed on ice in a solution containing 1% formaldehyde, 0.5% glutaraldehyde in PBS for 30 minutes. They were then rinsed in embryo buffer (PBS, 0.02% NP40, 0.01% deoxycholate, 2 mM $MgCl_2$) and incubated in staining solution (1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galacto-pyranoside (X-gal), 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$ and 1 mM ethylene glycol-bis(β-aminoethyl ether) N,N,N′,N′-tetraacetic acid (EGTA) in embryo buffer). After 3-4 hours at 37°C, or overnight at room temperature in the dark, the staining reaction was stopped by removing this solution and rinsing the embryos in PBS. The same procedure was followed for E10.5 and E13.5 blood staining, except that the cells were fixed for only 5 minutes.

In situ hybridization on erythroid cells

E10.5 embryonic blood from the heart or E13.5 liver cells from individual fetuses were disrupted in 50 and 150 μl of PBS respectively. 20 μl of this cell suspension was fixed onto a poly(lysine)-coated slide in 4% formaldehyde, 5% acetic acid for 20 minutes at room temperature. The cells were then washed 3× for 10 minutes in PBS and stored in 70% ethanol at −20°C. The remains after collection of blood or liver were stained for β-galactosidase to detect the transgenics.

Two probes were utilized for in situ hybridization. A nick-translated biotinylated *lacZ* probe for the detection of *lacZ* transcription, and DIG-labelled mouse α intron-specific oligonucleotides to reveal the erythroid cells. As a control, some of the slides were RNase-treated and hybridized with these probes. Hybridizations were done as described by Wijgerde et al. (1995) with a few modifications. The *lacZ* hybridization mix contained 50% formamide and 10% dextran sulphate. For antibody detection, the Tyramid amplification system TSA of Dupont was used (Raap et al., 1995).

Tissue culture and cell transfection

Murine erythroleukemia cells (MEL, strain C88) were maintained in DMEM plus 10% fetal calf serum. The construct μLCR-*lacZ* with neomycin as selectable marker was linearized at the *Sca*I site and transfected by electroporation as described (Antoniou et al., 1988; Antoniou and Grosveld, 1990). Stably transfected MEL populations were selected in G418 and induced to undergo erythroid differentiation by the addition of DMSO to a final concentration of 2% (v/v) for 2 days. The cells were collected and used for hypersensitive-site mapping. The DNaseI-treated samples were cut with *Eco*RI and *Sac*II. The Southern blots were hybridized with the 1.9 kb fragment of 5′HS3 (Talbot et al., 1989) or the *lacZ* probe to detect the hypersensitive sites.

RESULTS

Expression status of *lacZ* transgenes in mice as analyzed by X-gal staining

In order to analyze the expression status of the LCR and its individual hypersensitive sites (HS) with a ubiquitous promoter, each of these sites (Talbot et al., 1989) was linked to the minimal mouse *Hsp68* promoter. This 80 bp promoter contains a CCAAT box, an Sp1 binding site and a TATA box. All these constructs used the bacterial *β-galactosidase (lacZ)* gene as a reporter, to allow visualization of expression by X-gal staining (Bonnerot and Nicolas, 1993). These constructs were first tested in transgenic founder animals. Fetuses and yolk sacs were collected at 13.5 days of development (henceforth referred to as E13.5) and analyzed by X-gal staining All

Table 1. X-gal staining of transgenic founder fetuses at E13.5

Construct	Number of transgenic fetuses	X-gal staining in blood, yolk sac and fetal liver	Position effect
5′HS1-*lacZ*	5	2	3
5′HS2-*lacZ*	7	0	7
5′HS3-*lacZ*	6	5	1
5′HS4-*lacZ*	11	4	7
μLCR-*lacZ*	12	10	7

'Position effect' indicates ectopic expression, i.e. outside the erythroid system.

Table 2. X-gal staining of transgenic lines

Construct	Lines	X-gal staining in embryonic erythroid cells E8.5-E10.5	Position effect
5′HS1-*lacZ*	a	No	Yes
5′HS2-*lacZ*	a	No	Yes
	b	No	Yes
	c	No	Yes
	d	No	No
5′HS3-*lacZ*	a	Yes	Yes
	b	Yes	No
5′HS4-*lacZ*	a	No	Yes
	b	No	No
	c	No	Yes
	d	No	Yes
μLCR-*lacZ*	a	Yes	Yes
	b	Yes	No

'Position effect' indicates ectopic expression, i.e. outside the erythroid system.

fetuses were typed for the transgene by Southern blot hybridization. The results are summarized in Table 1.

LacZ expression was observed in blood, yolk sac and fetal liver of a few founder animals containing constructs with 5′HS1 or 5′HS4. However, no hematopoietic-specific staining was observed in founder fetuses with 5′HS2. In most of the 5′HS1, 5′HS2 and 5′HS4 founders, X-gal staining was observed in various other parts of the fetuses. This type of staining corresponds to classical position effects, to which the minimal *Hsp68* promoter is very sensitive (Kothary et al., 1987, 1988, 1989). These position effects show that the transgenes are functional and have not integrated in an inherently closed chromatin domain of the mouse genome. In sharp contrast, X-gal staining could be easily detected in the erythroid cells of the yolk sac, fetal liver and blood vessels in the majority of founder fetuses with either the μLCR or the 5′HS3 transgene. As expected, ectopic expression was also observed, since the LCR or parts of it do not neutralize position effects in non-erythroid tissues (Grosveld et al., 1987).

Previously, it has been shown that each of the hypersensitive sites of the LCR may have some developmental specificity (Fraser et al., 1993), and hence it was possible that the absence of erythroid staining in the 5′HS1, -2 and -4 founder fetuses is due to the stage specificity of these individual sites. Therefore, we studied the expression of the constructs in stable transgenic lines at different developmental stages. The data for each of the established stable transgenic lines are summarized in Table 2. F1 or F2 males from these lines were mated with non-transgenic females to collect embryos and fetuses at different developmental time points.

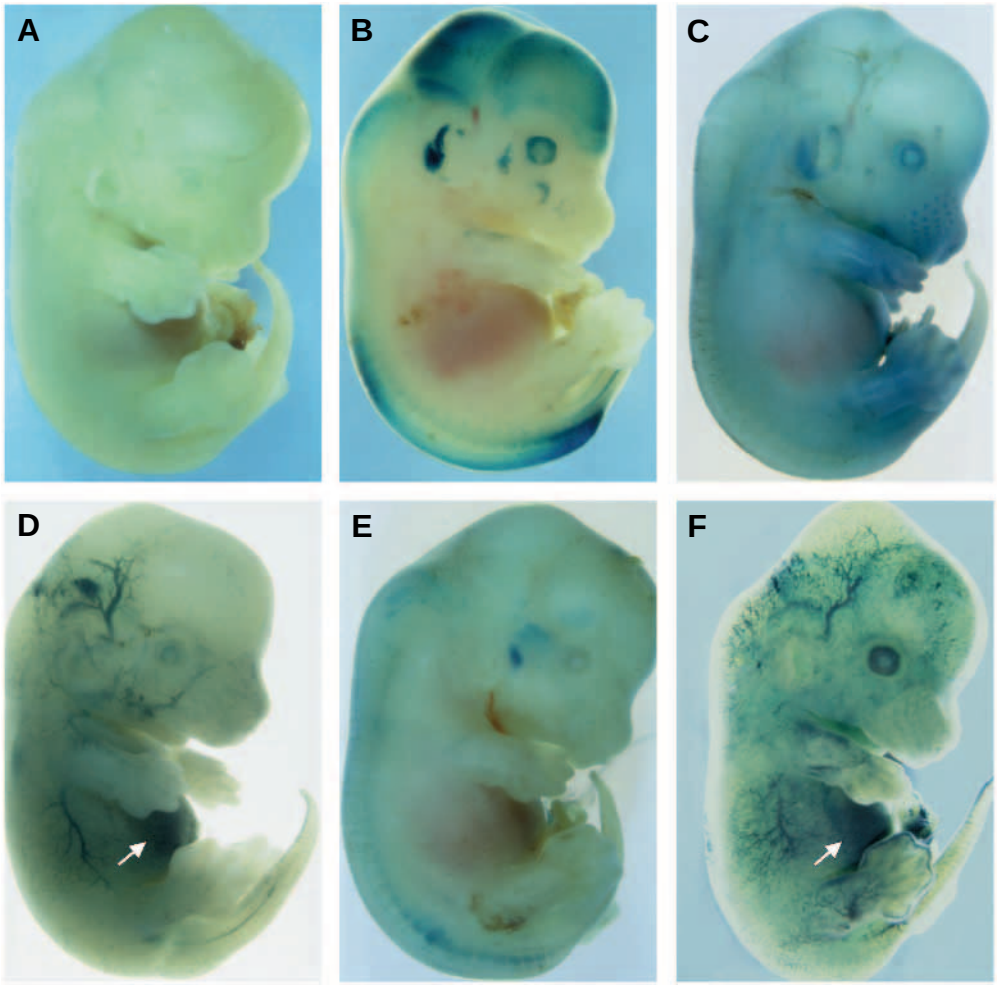

Fig. 1. X-gal-stained E13.5 transgenic fetuses from stable transgenic lines. (A) Control non-transgenic fetus; (B) 5′HS1-*lacZ*; (C) 5′HS2-*lacZ*; (D) 5′HS3-*lacZ*; (E) 5′HS4-*lacZ*; and (F) μLCR-*lacZ*. The fetal liver is indicated with an arrow in D and F. Ectopic expression, i.e. expression outside the erythroid system, can be seen in B, C and E. Original magnification, 8×.

These results revealed that, at E13.5, X-gal staining of the erythroid cells could only be detected in the transgenic lines containing the µLCR or 5′HS3 constructs (Fig. 1D,F). This is in agreement with the data obtained from the founder fetuses (Table 1). Staining of the erythroid system was not seen for the other constructs (Fig. 1B,C,E). Again, expression outside the erythroid system, due to position effects, was observed in many of the lines with all of the constructs (Fig. 1 and Tables 1, 2).

The expression status of the transgenes at earlier stages in mouse development was examined by X-gal staining of embryos at various time points starting from the onset of hematopoiesis at E7.5-8. Embryos containing the 5′HS1, 5′HS2 and 5′HS4 transgenes showed no staining in the yolk sac or blood cells at any of the stages examined (Table 2). However, the µLCR and 5′HS3 lines showed the first erythroid-specific staining at E8-8.5, with a ring of blue cells observed at the base of the yolk sac (Fig. 2B, arrow). Later, X-gal staining cells are found over the entire yolk sac (Fig. 2C,D). In E9-E10.5 embryos, the staining is seen in the cells within the blood capillaries (Fig. 2E-G), the heart (Fig. 2G, arrows) and the dorsal aorta (Fig. 2G, arrowheads). In addition, staining of the fetal liver is observed in E13.5 fetuses (Fig. 1D,F, arrows). Surprisingly, very few blue cells are found in adult blood from the same transgenic lines (Fig. 3A). This prompted us to study the blood from E10.5 and E13.5 transgenics in more detail. While >98% of the cells are *lacZ*-positive in E10.5 blood (Fig. 3F), this number decreases to around 50% in E13.5 blood (Fig. 3E). Most of the cells staining at the early stages are presumably embryonic primitive erythroid cells, since they are large and nucleated (Fig. 3E,F). Strikingly, less than 10% *lacZ*-positive cells are found in the fetal liver (Fig. 3C), the site of erythropoiesis at E13.5 (see Fig. 1D,F, arrows). These data therefore suggest that the µLCR and 5′HS3 transgenes are active in primitive cells of embryonic origin, but not in fetal- and adult-type definitive erythroid cells.

DNaseI hypersensitive site mapping in the fetal liver of *lacZ* transgenic mice

The LCR is characterized by the presence of developmentally stable, very strong, erythroid-specific DNaseI hypersensitive sites (Grosveld et al., 1987; Jimenez et al., 1992b; Tuan et al., 1985). To determine whether these hypersensitive sites are correctly established in our transgenic mice, we examined the chromatin structure in E13.5 liver of the *lacZ* transgenic lines described above. Nuclei from the livers of E13.5 fetuses were

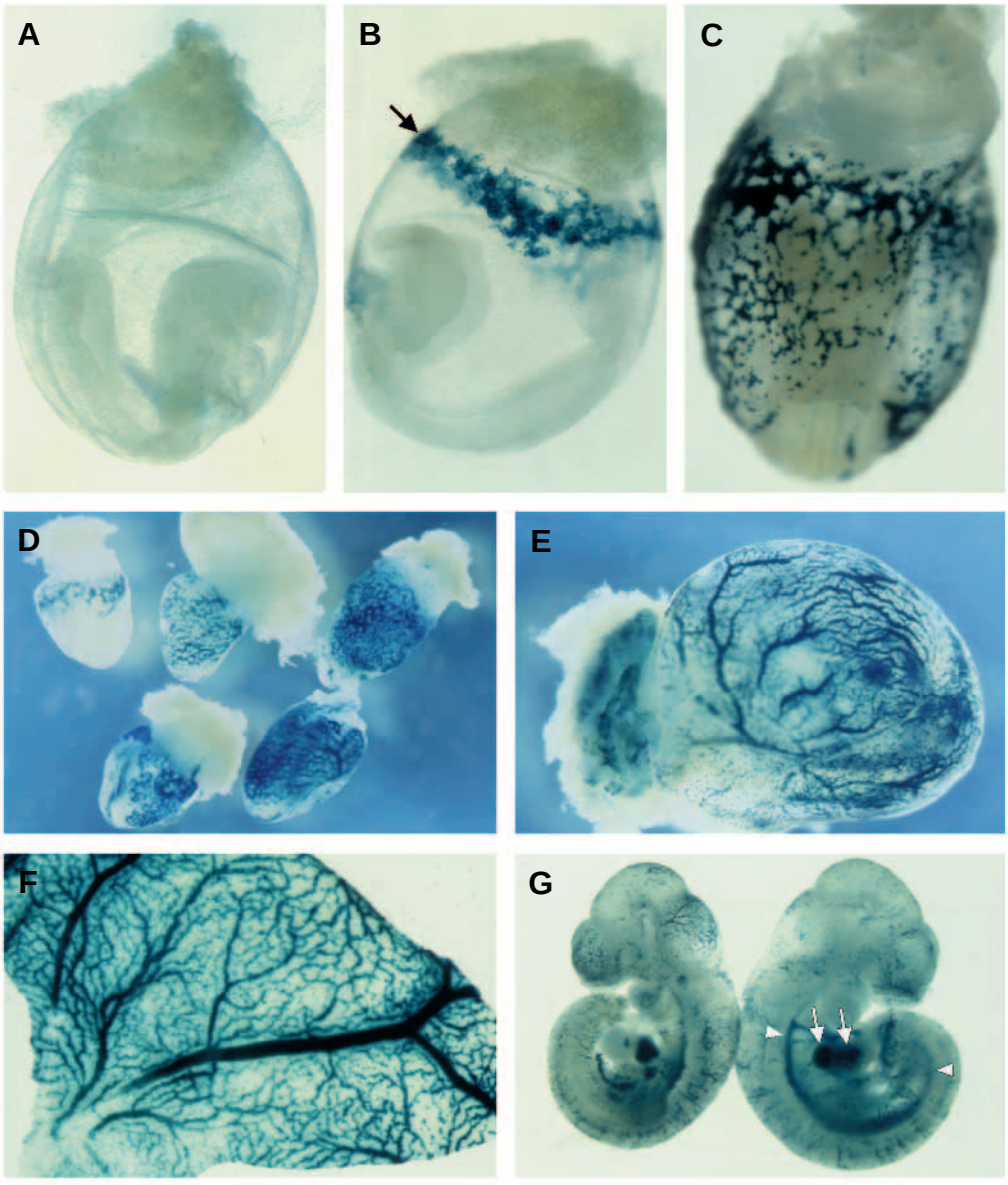

Fig. 2. Developmental (E8-E11.5) X-gal staining pattern in the 5′HS3-*lacZ* line b. (A) Non-transgenic E8 embryo. (B) E8 transgenic embryo; the ring of blue cells at the base of the yolk sac is indicated by an arrow. (C) E8.5 transgenic embryo. (D) Various stages observed in E8-8.5 transgenic embryos. (E) E9.5 transgenic embryo enclosed in the yolk sac. (F) E10.5 yolk sac staining. (G) E10.5 (left) and E11.5 (right) transgenic embryos; the heart is indicated by arrows, the dorsal aorta by arrowheads. Original magnification, 80× (A,B,C,F); 25× (D,G); 50× (E). Data are shown for the 5′HS3-*lacZ* line b in Figs 2, 3 and 5, but it should be noted that identical results were obtained with the other 5′HS3-*lacZ* and both of the µLCR-*lacZ* lines.

treated with DNaseI (Ellis et al., 1996), after which the purified DNA was digested with *Eco*RI and Southern-blotted. As a control, the Southern blots were first hybridized with a probe detecting the hypersensitive site at the mouse β-major promoter to assess DNaseI sensitivity. The blots were then hybridized with a *lacZ* probe to detect hypersensitive sites at the transgenes. As a reference, tail DNA from each transgenic mouse line was cut with *Eco*RI and *Nco*I to show the position of the *Hsp68* promoter. To mark the positions of the core elements of the LCR fragments, *Eco*RI-digested tail DNA was recut with *Spe*I for 5′HS1, *Hin*dIII for 5′HS2, *Apa*I for 5′HS3 and *Sph*I for 5′HS4 (Talbot et al., 1989).

The results obtained from this analysis on E13.5 liver show that hypersensitive sites are not detectable at the correct positions within any of the transgenes, while the hypersensitive site at the mouse β-major promoter could be detected consistently (Fig. 4). For example, the weak sensitivity seen in DNA from the 5′HS2-*lacZ* line b (grey arrow in Fig. 4D) does not map to the core fragment of 5′HS2, nor to the *Hsp68* promoter and most likely reflects the general, non-erythroid, DNaseI sensitivity associated with 5′HS2 (e.g. Ellis et al., 1996). These results are not surprising for the lines with the 5′HS1, -2 and -4 constructs, since we could not detect erythroid-specific expression of the *lacZ* gene in these mice. However, no DNaseI hypersensitivity is observed in the DNA from the lines with the μLCR and 5′HS3 constructs (Fig. 4B,E), even though *lacZ* protein is easily detectable at this stage (Fig. 1D,F; Fig. 3C). Since it is possible that the *lacZ* gene was transcribed only at earlier stages in fetal liver development, we repeated the DNaseI fade-out analysis on E12.5 livers of the μLCR and 5′HS3 lines. These results were also negative (data not shown) and thus, we conclude that the transgenes are in a closed chromatin conformation in the fetal liver.

In situ hybridization to detect active transcription of the transgene

Since the numbers of erythroid cells that can be obtained from E8.5-E10.5 embryos are too small for a DNaseI sensitivity analysis (N. Gillemans, unpublished results), we used in situ hybridization as an alternative to monitor the activity of the μLCR and 5′HS3 transgenes. To more closely examine when and at what frequency the μLCR- and 5′HS3-*lacZ* transgenes are actively transcribed, we analyzed specific transcription of the *lacZ* gene at the single cell level (Wijgerde et al., 1995). In situ hybridization was performed on erythroid cells from E10.5 blood and E13.5 liver with a biotin-labelled *lacZ* probe to detect transcription of the transgenes. Oligonucleotide mouse α-globin probes were used to establish the number of erythroid cells in each preparation. The slides were analyzed by confocal-or standard fluorescence microscopy.

When *lacZ* was used as probe, we observed a single nuclear signal in a significant number (around 15%) of cells in E10.5 blood (Table 3; Fig. 5A, arrow). The much higher percentage

Table 3. Percentage of cells actively transcribing the *lacZ*- or the endogenous mouse *α-globin* genes as determined by in situ hybridization

Time points	Cell type	Line	Percentage of cells with *lacZ* nuclear signal	Percentage of cells with mouse *α-globin* nuclear signals
E10.5	Blood	5′HS3-*lacZ* b	16	92
		μLCR-*lacZ* b	15	97
E13.5	Fetal liver	5′HS3-*lacZ* b	0.5	97
		μLCR-*lacZ* b	1.1	95

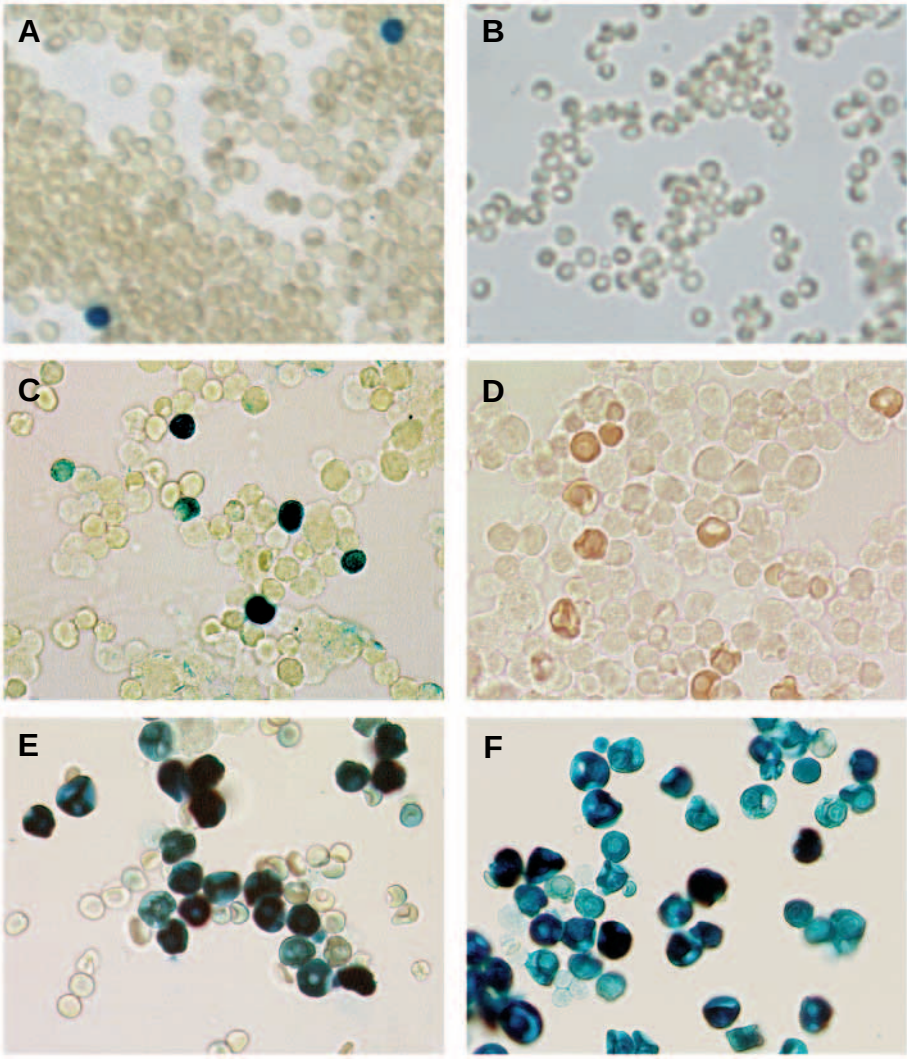

Fig. 3. X-gal staining of blood and fetal liver cells from the 5′HS3-*lacZ* line b. (A) Transgenic adult blood; (B) non-transgenic adult blood; (C) E13.5 transgenic liver cells; (D) E13.5 non-transgenic liver cells; (E) E13.5 transgenic blood; and (F) E10.5 transgenic blood. Original magnification, 400×.

of X-gal staining cells (Fig. 3F) suggests that the *lacZ* gene may not be transcribed continuously, as has been observed in transgenic mice carrying the human *β-globin* locus with small deletions in the LCR (Milot et al., 1996). Hence, we conclude that the hemizygous *lacZ* gene is actively transcribed at this stage. Contrasting observations were made with E13.5 liver cells where only a few cells (0.5-1%) had a nuclear signal (Table 3 and Fig. 5B). The control hybridization for expression of the homozygous mouse *α-globin* genes detected two nuclear signals in >90% of the cells (Table 3; Fig. 5C, arrowheads), confirming that these cells are of the erythroid lineage. No nuclear signal was detected in RNase-treated or *lacZ*- negative cells at either stage when hybridized with the *lacZ* probe (Fig. 5D and data not shown). Thus, we conclude that at E10.5, many of the erythroid cells from the μLCR and 5′HS3 lines are actively transcribing the *lacZ* gene. As development advances, the percentage of actively transcribing cells becomes considerably lower. This is in agreement with our DNaseI fade-out analysis, in which <1% of cells with an active gene in E13.5 liver would be too low to detect hypersensitive sites at the transgene.

DNaseI hypersensitive site mapping in MEL cell populations with μLCR-*lacZ*

The absence of hypersensitive sites in the μLCR-*lacZ* and 5′HS3-*lacZ* transgenes raised the question whether these constructs are intrinsically inactive in the adult erythroid environment. To investigate this, we transfected the μLCR-*lacZ* construct into murine erythroleukemia (MEL) cells. These cells represent the pro-erythroblast stage of adult cell differentiation. Stable clones were obtained by selection with G418, thus assuring integration into an active area of the chromatin. The cell populations were expanded and induced with DMSO to undergo erythroid differentiation for 2 days. These cells were then analyzed for DNaseI sensitivity, as described earlier (Philipsen et al., 1990). The blots were hybridized with a 5′HS3 probe, because the *lacZ* probe hybridizes to vector sequences precluding a straightforward interpretation of the results (not shown). Interestingly, we find that all four DNaseI hypersensitive sites are strongly represented, as shown in Fig. 6. The sub-band corresponding to the hypersensitive site at 5′HS4 runs just below the parent restriction band and is not well resolved. However, the hypersensitive sites at 5′HS1 and the promoter (which comigrate), 5′HS2 and 5′HS3 are found. Thus, we conclude that the μLCR-*lacZ* construct is not intrinsically inactive in the adult erythroid environment.

DISCUSSION

Activation of *lacZ* transgenes by the β-globin LCR

Previous studies involving the human β-globin LCR and its individual hypersensitive sites 5′HS1-4 have shown that these regulatory elements confer position-independent, copy-number dependent expression to the *β-globin* gene in transgenic mice (Caterina et al., 1991, 1994; Ellis et al., 1993; Fraser et al., 1990; Philipsen et al., 1990, 1993; Pruzina et al., 1991, 1994; Ryan et al., 1989; Talbot et al., 1990). It has not been established whether the LCR can direct erythroid-specific expression in the absence of any globin gene sequences. We linked the μLCR or individual HS elements to a simple 80 bp promoter derived from the mouse *Hsp68* gene that does not have any developmental or tissue specificity (Kothary et al., 1987, 1988, 1989).

We analyzed the ability of the μLCR and 5′HS1, 5′HS2,

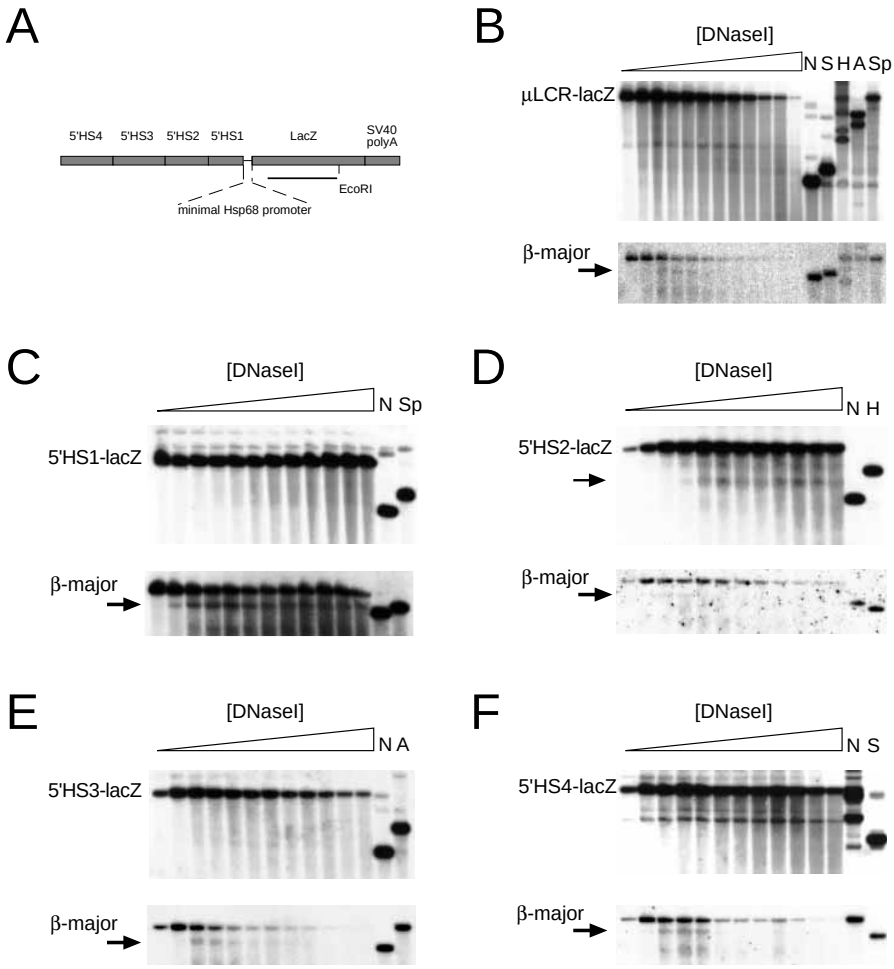

Fig. 4. DNaseI hypersensitive site mapping. (A) Schematic drawing of the μLCR-*lacZ* construct. The probe is indicated with a bar. (B-F) Hypersensitive site analysis in e13.5 liver of transgenic lines. The DNA was digested with *Eco*RI (see Materials and Methods). For each line, a control hybridization detecting the hypersensitive site at the promoter of the mouse β-major gene is shown below. The triangles indicate increasing amounts of DNaseI. Hypersensitive sites are indicated by arrows. (B) μLCR-*lacZ* line b; (C) 5′HS1-*lacZ* line a; (D) 5′HS2-*lacZ* line b; (E) 5′HS3-*lacZ* line b; (F) 5′HS4-*lacZ* line a. Control lanes contain DNA digested with *Eco*RI plus another enzyme to mark the position of the *Hsp68* promoter or the LCR regions where hypersensitive sites should appear: N, *Nco*I (*Hsp68* promoter), Sp, *Spe*I (5′HS1), H, *Hin*dIII (5′HS2), A, *Apa*I (5′HS3), S, *Sph*I (5′HS4).

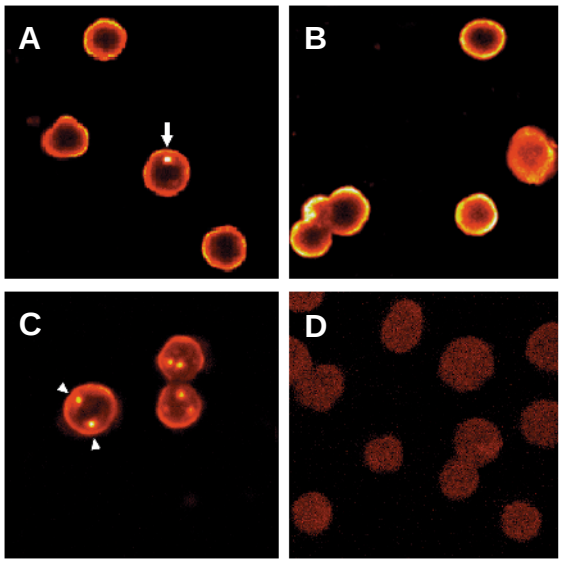

Fig. 5. In situ hybridization to detect active transcription of the *lacZ* transgene in the 5′HS3-*lacZ* line b. (A) E10.5 embryonic blood hybridized with the *lacZ* probe. A single nuclear signal corresponding to active transcription of the hemizygous *lacZ* gene is indicated by an arrow. (B) E13.5 fetal liver cells hybridized with the *lacZ* probe; there are virtually no cells with nuclear signal (Table 3). (C) Control hybridization with oligonucleotide probes detecting transcription of the homozygous mouse *α-globin* gene; two transcription foci per nucleus are indicated by arrowheads. (D) E10.5 blood from a control non-transgenic mouse hybridized with the *lacZ* probe. Original magnification, 630×.

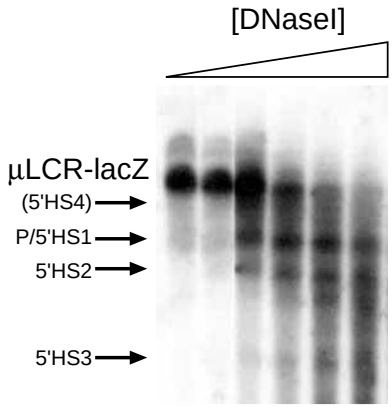

Fig. 6. Hypersensitive site mapping of the μLCR-*lacZ* construct in MEL cells. MEL cells were stably transfected with the μLCR-*lacZ* construct, and hypersensitive site analysis was performed as described (Philipsen et al., 1990). The DNA was digested with *Eco*RI and *Sac*II, Southern blotted and hybridized with the 1.9 kb 5′HS3 fragment [see Fig. 4A; Talbot et al. (1989)]. Other details are as in Fig. 4.

5′HS3 and 5′HS4 to activate this reporter construct in the erythroid cells of transgenic mice. The results show that only the complete μLCR and 5′HS3 can direct expression of the *lacZ* reporter in the erythroid system of the developing mouse. While erythroid-specific staining was observed in some of the E13.5 founder fetuses analyzed for 5′HS1 and 5′HS4, none of the established transgenic lines with these constructs showed such a staining at any stage of development. This indicates that 5′HS1 and 5′HS4 need other elements to activate erythroid-specific transcription.

5′HS2 is believed to be a crucial component of the LCR. This part of the LCR shows the highest degree of evolutionary conservation (Hardison et al., 1993; Jimenez et al., 1992a). Its function has been studied in great detail in transfection (Hardison et al., 1993; Moon and Ley, 1991; Tuan et al., 1989), transgenic (Caterina et al., 1991, 1994; Ellis et al., 1993, 1996; Ryan et al., 1989; Talbot et al., 1990) and knockout experiments (Fiering et al., 1995). To our surprise, we could not detect any erythroid-specific expression with the construct containing 5′HS2, neither in E13.5 day founders, nor at any stage of development in four established lines. Thus we conclude that 5′HS2 is also unable to activate the non-erythroid *Hsp68* promoter in the erythroid lineage. The frequently observed ectopic expression (i.e. expression outside the erythroid cells) in 5′HS2 transgenics shows that this construct is not intrinsically inactive. Rather, these results suggest that 5′HS2 is dependent on local elements provided by the *β-globin* gene for erythroid-specific activation. Preliminary data show that these elements might reside within the first 800 bp of the β-globin promoter, since we have one E13.5 founder with 5′HS2 and the 5′-800 β-globin promoter driving the *lacZ* gene that shows extensive staining of the erythroid system (not shown).

It should be noted that all the transgenes were present as multi-copy concatamers. Previous studies from our laboratory have shown that only 5′HS3 of the LCR is capable of activating the *β-globin* gene in single-copy transgenic mice. 5′HS2 was only active when present in a multi-copy configuration (Ellis et al., 1996). In contrast, it appears from the present study that 5′HS2 is not able to activate the minimal *Hsp68* promoter, even in multi-copy transgenics. Our data corroborate those of Ellis et al. (1996) that 5′HS3 is the most important hypersensitive site of the β-globin LCR.

Developmental expression pattern of the *lacZ* transgenes

In the lines with the μLCR or 5′HS3, expression of the *lacZ* transgene could first be detected at E8-8.5 as a ring of cells around the base of the yolk sac. This is the first site of erythropoiesis in the developing mammalian embryo (Russell and Bernstein, 1966). Later, as vascularization occurs, *lacZ*-positive cells are found in the developing circulatory system of the embryo. In E10.5-11 blood, the vast majority of the circulating primitive erythrocytes are *lacZ*-positive. A paradox occurs in E13.5 liver. Although the erythroid fetal liver can be easily stained for the presence of the *lacZ* protein, large numbers of erythrocytes begin to appear in the circulation that do not stain with X-gal. We believe that these data can be explained by the model for the development of the hematopoietic system proposed by Dzierzak and Medvinsky (1995). According to this model, the fetal liver is first colonized by a wave of primitive hematopoietic precursor cells derived from the yolk sac. A second wave of colonization occurs when definitive stem cells originating from the AGM (Aorta, Gonads, Mesonephros) region migrate to the fetal liver. In our transgenic mice, the *lacZ*-positive cells in the fetal liver would be derived from the yolk sac precursor cells. These cells actively expressed the transgene at the embryonic stages of development. Although they have switched off the *lacZ* gene in the E13.5 fetal liver, as we have shown by in situ hybridization, they still contain *lacZ* protein and mRNA. Hence, these cells may stain *lacZ*-positive even after a number of cell divisions. The *lacZ*-negative cells

in the circulation would be derived from fetal liver cells originating from the AGM region, which have never expressed the *lacZ* gene at detectable levels. This model implies that the *lacZ* gene has a repressive effect on the LCR in definitive but not in primitive erythropoiesis. Recent observations of Guy et al. (1996) and Robertson et al. (1995) are consistent with this hypothesis, and preclude the simple explanation that our observations are an artefact of the particular transgene construct that we have used. These authors found that the presence of the *lacZ* gene, even in the presence of a complete *β-globin* gene (Guy et al., 1996), interferes with LCR function (Robertson et al., 1995). Unfortunately, these groups did not present data on early embryonic time points. The absence of DNaseI hypersensitive sites in E13.5 fetal liver suggests that the repression is due to heterochromatization of the transgenes (Adams and Workman, 1993; Elgin, 1990; Hebbes et al., 1992; Patterton and Wolffe, 1996), and not caused by the inability of the LCR to interact with the model promoter we employed. Recent data of knockout experiments in the mouse *β-globin* locus show that nonerythroid promoters can be activated very efficiently by the LCR (Fiering et al., 1995; Hug et al., 1996; Kim et al., 1992; Nandi et al., 1988; Shehee et al., 1993). Consistent with these observations, we can detect hypersensitive sites at the transgene when transfected into MEL cells. Mechanistically, the *lacZ* gene could serve as a nucleation point for heterochromatization in definitive erythroid cells, resulting in repression of the transgenes in mice. In tissue culture, these events are eliminated because the selection procedure only leaves cells in which integration has occurred in open areas of chromatin, to allow expression of the resistance marker. The results suggest that the μLCR-*lacZ* construct is active when integrated in such chromatin in adult erythroid cells, but that it is suppressed when it integrates in closed chromatin domains, as is probably the case in transgenic mice. The reason for the inactivation in mice is presently unknown, but we have ruled out the possibility that it is caused by progressive methylation of the transgenes (results not shown).

Previous studies have shown that the μLCR and 5′HS3 direct high level expression of the *β-globin* gene in transgenic mice (Ellis et al., 1996; Philipsen et al., 1990, 1993; Talbot et al., 1989); the data in this paper indicate that the presence of this gene is a requirement for the activity of the μLCR and 5′HS3 in fetal and adult erythroid cells.

Hypersensitive site formation in the LCR

Our data are in apparent contradiction with a number of published observations regarding hypersensitive site formation in the LCR. Two independent laboratories have studied this for 5′HS2 and 5′HS4. In the case of 5′HS2, Pawlik et al. (1995) have shown that 5′HS2, in the absence of a gene, has a DNaseI-hypersensitive site in transgenic mice. We also observe weak hypersensitivity at 5′HS2 in our transgenic lines, and suggest that this corresponds to the ubiquitous hypersensitive site associated with the human 5′HS2 (Ellis et al., 1996). This hypersensitive site is located outside the core fragment of 5′HS2, where the erythroid-specific activity resides.

Transgenic mice experiments also suggest that 5′HS4 can autonomously form a hypersensitive site (Lowrey et al., 1992). However, since only one transgenic line was analyzed in this study, the transgene may have integrated in an area of open chromatin. Stamatoyannopoulos et al. (1995) have reported on the formation of 5′HS4 in the presence of 5′HS3 in transfection experiments in MEL cells. As we show here, we can detect hypersensitive sites when we transfect the μLCR-*lacZ* construct into MEL cells, but not in the transgenic lines.

Our data are compatible with those obtained for the chicken *β-globin* locus in transgenic mice. These data suggest that hypersensitive site formation in the chicken 3′ enhancer is dependent on the presence of an active promoter (Reitman et al., 1993). We have shown that in E13.5 fetal liver the μLCR and 5′HS3 transgenes are in a closed chromatin conformation, and that transcription of the *lacZ* gene is not detectable in these cells. Hence, as in the chicken case (Reitman et al., 1993), a closed promoter correlates with the absence of hypersensitive sites in the LCR.

Perspective

The data in this paper show that the μLCR and 5′HS3 can activate the minimal *Hsp68* promoter in transgenic mice. The other HS elements of the LCR are inactive and therefore require additional elements of the *β-globin* locus for their activity. The expression of the μLCR and 5′HS3 transgenes is specific for embryonic, primitive erythropoiesis. Hence, these mice are useful tools for the study of these first erythroid cells. Recently, gene-targeting experiments have implicated a number of transcription factors in the development of the erythroid cell (reviewed in Shivdasani and Orkin, 1996). The role of these transcription factors in the activating function of the μLCR and 5′HS3 in primitive erythropoiesis can now be addressed directly by breeding our transgenes into these knockout backgrounds.

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