Erythroid Krüppel-like factor (EKLF) is active in primitive and definitive erythroid cells and is required for the function of 5'HS3 of the β -globin locus control region

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Disruption of the gene for transcription factor EKLF (erythroid Krüppel-like factor) results in fatal anaemia caused by severely reduced expression of the adult β -globin gene, while other erythroid-specific genes, including the embryonic ϵ - and fetal γ -globin genes, are expressed normally. Thus, EKLF is thought to be a stage-specific factor acting through the CACC box in the β -gene promoter, even though it is already present in embryonic red cells. Here, we show that a β -globin gene linked directly to the locus control region (LCR) is expressed at embryonic stages, and that this is only modestly reduced in EKLF^{-/-} embryos. Thus, embryonic β -globin expression is not intrinsically dependent on EKLF. To investigate whether EKLF functions in the locus control region, we analysed the expression of LCR-driven *lacZ* reporters. This shows that EKLF is not required for reporter activation by the complete LCR. However, embryonic expression of reporters driven by 5'HS3 of the LCR requires EKLF. This suggests that EKLF interacts directly with the CACC motifs in 5'HS3 and demonstrates that EKLF is also a transcriptional activator in embryonic erythropoiesis. Finally, we show that overexpression of EKLF results in an earlier switch from γ - to β -globin expression. Adult mice with the EKLF transgene have reduced platelet counts, suggesting that EKLF levels affect the balance between the megakaryocytic and erythroid lineages. Interestingly, the EKLF transgene rescues the lethal phenotype of EKLF null mice, setting the stage for future studies aimed at the analysis of the EKLF protein and its role in β -globin gene activation.

Keywords: EKLF/ β -globin/locus control region/primitive erythropoiesis

Introduction

The human β -globin gene cluster contains five functional genes 5'- ϵ (embryonic)-G γ -A γ (fetal)- δ - β (adult)-3'. Expression of these genes is completely dependent on the

presence of the locus control region (LCR) upstream of the ϵ -globin gene. The LCR contains five DNaseI hypersensitive sites, termed 5'HS1–5, distributed over 20 kb, and a fully functional 6.5 kb version of the LCR containing 5'HS1–4 (μ LCR) has been described (Talbot *et al.*, 1989). The functional activity of the 5'HSs resides in 200–300 bp 'core' fragments coinciding with the hypersensitive areas (reviewed in Grosveld *et al.*, 1993).

Erythroid Krüppel-like factor (EKLF) is an erythroid-specific transcription factor containing three archetypal zinc fingers (Miller and Bieker, 1993). It binds to the DNA sequence CCA CAC CCT, which is found in the promoter of the adult β -globin gene, and related sequences (Feng *et al.*, 1994). Naturally occurring point mutations in this promoter element, also known as the CACC box, result in reduced expression of the β -globin gene linked *in cis* (reviewed in Thein, 1993), suggesting that EKLF is an important activator of β -globin gene expression. This notion was confirmed by the phenotype of EKLF knock-out mice (Nuez *et al.*, 1995; Perkins *et al.*, 1995). These mice die at around day 14–15 of gestation (E14–15) due to anaemia caused by failure to express the β -globin gene.

The role of EKLF in the expression of the human β -globin gene cluster has been analysed by crossing mice carrying the complete human locus into the EKLF null background (Perkins *et al.*, 1996; Wijgerde *et al.*, 1996). This showed that the embryonic ϵ - and the fetal γ -globin genes are expressed at normal levels in EKLF^{-/-} fetuses, but expression of the adult β -globin gene is barely detectable. Thus, these data suggest that EKLF exerts its effect on β -globin expression through the CACC box in the promoter of this gene. Hypersensitivity analysis showed that the 5'HS3 region of the LCR was also affected. It contains CACC box motifs, suggesting that the loss of hypersensitivity is a direct result of the absence of EKLF. However, the CACC box is also present in the promoters/enhancers of many other erythroid-specific genes including the α -globins (Philipsen *et al.*, 1990) which are not affected. Thus, the loss of hypersensitivity could be an indirect effect caused by changes in the interactions of the LCR with the β -globin gene (Wijgerde *et al.*, 1996).

In the developing mouse embryo, the first erythroid cells are derived from the yolk sac and start to appear at E7.5. These cells remain nucleated and are referred to as primitive cells. The first enucleated erythrocytes are formed in the fetal liver at around E11.5; both fetal and adult erythrocytes are referred to as definitive cells. There is good evidence that primitive and definitive cells arise from two independent lineages in the embryo (reviewed in Dzierzak and Medvinsky, 1995). Interestingly, the EKLF protein is already present in embryonic erythroid cells (Southwood *et al.*, 1996), but as yet, no defect in primitive erythropoiesis has been reported for EKLF^{-/-} mice (Nuez

et al., 1995; Perkins *et al.*, 1995). Thus, it would appear that EKLK is not active in primitive cells.

In this paper, we have investigated the activity of EKLK in the developing erythroid system and its potential role in the LCR. First, we demonstrate that a β -globin gene linked directly to the μ LCR is expressed at the embryonic stage and that embryonic expression is only moderately affected in EKLK^{-/-} embryos. Thus, expression of the β -globin gene is not intrinsically dependent on EKLK in primitive cells. Secondly, we investigated the link between EKLK and LCR activity in the absence of β -globin gene sequences through the use of μ LCR-*lacZ* mice (Tewari *et al.*, 1996). We show that embryonic expression of the μ LCR-*lacZ* construct is retained in the EKLK null background. In contrast, the expression of *lacZ* constructs with only 5'HS3 or the core fragment of 5'HS3 is dependent on EKLK in primitive cells, demonstrating that EKLK is also active in primitive cells and required for the activity of 5'HS3 of the LCR. Next, we investigated the effect of increased EKLK levels on γ - to β -globin switching by expressing EKLK cDNA under the control of the β -globin LCR and promoter. The results show that increased EKLK levels change the balance from γ - to β -globin gene expression in definitive cells, suggesting that the amount of EKLK influences the rate of the switching process. We further used the EKLK transgene to rescue the EKLK null mutation. These rescue mice had almost normal haematological parameters, with slightly reduced haemoglobin levels due to a reduction in the number of erythrocytes. Interestingly, wild-type mice with the EKLK transgene have reduced numbers of circulating platelets, suggesting that EKLK may have a role in determining the balance between the megakaryocytic and erythroid lineages.

Results

Expression of the β -globin gene in primitive erythropoiesis

A β -globin gene linked directly to the LCR is expressed prematurely in embryonic erythroid cells (Hanscombe *et al.*, 1991; Dillon *et al.*, 1997). The EKLK protein is known to be present in these cells (Southwood *et al.*, 1996) but no function has been assigned to EKLK in primitive erythropoiesis (Nuez *et al.*, 1995; Perkins *et al.*, 1995); a Northern blot showing the absence of EKLK-encoding mRNA in E10.5 α -yolk sac RNA is shown in Figure 1A. Thus, we wondered whether embryonic expression of the β -globin gene is also dependent on EKLK. To test this directly, we used a transgenic line with a single copy of the μ LCR- β -globin construct (Figure 1B). In E13.5 fetal liver, this human β -globin transgene is expressed at the same level as the endogenous β -major gene (line μ D14 in Ellis *et al.*, 1996). First, we determined transgene expression in primitive cells. We isolated RNA from E10.5 embryos and quantitated the expression level of the human β -globin gene relative to that of the endogenous mouse α -globin gene, which is expressed in both primitive and definitive cells and is not affected by the EKLK knockout. The results are shown in Figure 1C. As could be predicted, the β -globin gene is expressed prematurely due to the absence of the ϵ - and γ -globin genes in the construct (Hanscombe *et al.*, 1991; Dillon *et al.*, 1997). We then

analysed expression of the transgene in EKLK^{-/-} embryos. Interestingly, there is only a modest (2- to 3-fold), reduction in the level of transgene-derived mRNA in E10.5 EKLK^{-/-} embryos, while expression of the transgene is more drastically affected in E13.5 EKLK^{-/-} fetal liver (>6-fold reduction). This reduction is smaller than reported previously for adult β -globin expression (Perkins *et al.*, 1996; Wijgerde *et al.*, 1996) which could be the result of the presence of human β -globin mRNA in circulating primitive cells. We therefore performed primary transcript *in situ* hybridization to determine the percentage of cells actively transcribing the transgene (Wijgerde *et al.*, 1995). Probes detecting transcription of the mouse α -globin genes were used as a control to establish the number of erythroid cells in each preparation. Figure 1D shows that the β -globin transgene is actively transcribed in >95% of the cells in EKLK^{+/+} E10.5 blood (Table I). In the absence of EKLK, active transcription of the human β -globin gene is observed in ~55% of the cells, in good agreement with the S1 nuclease analysis. Interestingly, human β -globin gene transcription is detected in <1% of the cells in EKLK^{-/-} fetal liver, while 95% of E13.5 EKLK^{+/+} fetal liver cells actively transcribe the transgene (Figure 1D and Table I). The extremely low number of cells expressing the transgene in EKLK^{-/-} mice closely resembles previous observations on adult β -globin gene transcription (Wijgerde *et al.*, 1996). Thus, the relatively high level of human β -globin mRNA in E13.5 fetal liver of EKLK^{-/-} mice observed by S1 nuclease analysis is due to earlier expression of the transgene in primitive erythroid cells still present in the circulation at E13.5.

We conclude that EKLK has only a modest effect on the expression of the human β -globin transgene in primitive cells, implying that expression of the β -globin gene is not intrinsically dependent on EKLK in this lineage. However, EKLK is essential for β -globin expression in definitive cells, regardless of the presence of embryonic and fetal globin genes.

EKLK is a transcriptional activator in primitive erythroid cells required for the function of 5'HS3

The above results gave the first indication that EKLK could function as a transcriptional activator in the primitive lineage, since we observed a reduced expression of the β -globin transgene in EKLK^{-/-} primitive cells. To further study the potential role of EKLK in LCR-mediated gene activation in embryonic erythroid cells, we used *lacZ* reporter mice in which μ LCR constructs drive expression of the *lacZ* gene from a basic, non-erythroid promoter (Tewari *et al.*, 1996). Three different constructs were used (Figure 2A). The first contained the μ LCR, as used in the μ LCR- β -globin construct described above. The second construct contained the 1.9 kb 5'HS3 fragment of the μ LCR, and the third construct contained only the 225 bp core fragment of 5'HS3 with six potential EKLK binding sites (Philipsen *et al.*, 1993; Philipsen *et al.*, 1990). *LacZ* expression of the μ LCR and 5'HS3 transgenes is confined to primitive cells, while the core 5'HS3 transgene is also expressed in definitive erythroid cells (not shown; Tewari *et al.*, 1996).

The three reporter lines were bred into the EKLK^{-/-} background, and expression of the *lacZ* reporter gene in primitive cells was assayed by X-gal staining of E10.5

Fig. 1. Expression of the μ LCR- β -globin transgene in EKLF null mice. (A) Absence of EKLF mRNA in E10.5 $EKLF^{-/-}$ yolk sac. Total RNA was isolated from E10.5 day yolk sacs, subjected to Northern blotting and hybridized with EKLF cDNA to demonstrate the absence of EKLF encoding mRNA in the knock-out samples (top). To control for loading of the gel, the same blot was rehybridized with a cDNA encoding the haem synthesis enzyme ALA-S (bottom). (B) The human β -globin locus and the μ LCR- β -globin construct. The μ LCR was directly linked to the human β -globin gene, and this construct was used to generate a single copy transgenic mouse line (μ D14 in Ellis *et al.*, 1996). (C) The expression of the μ LCR- β -globin transgene in primitive and definitive cells of $+/+$, $+/-$ and $-/-$ EKLF fetuses. The fetuses were dissected at the stages indicated, genotyped, and RNA was isolated from the whole embryo (E10.5) or the fetal liver (E13.5). Expression of the transgene was determined with the S1-nuclease protection assay; the endogenous mouse α -globin mRNA was used as an internal control. The results were quantitated on a PhosphorImager. The specific activity of the human β -globin probe was approximately six times higher than that of the mouse α -globin probe. (D) Primary transcript *in situ* hybridization of transgene expression in EKLF knock-out mice. Cells were isolated from E10.5 blood or E13.5 fetal liver and subjected to *in situ* hybridization to detect active transcription of the β -globin transgene (Wijgerde *et al.*, 1995). Genotypes of the fetuses are as indicated. Representative examples of cells with signals corresponding to α -globin (green) and human β -globin (red) are shown.

Table 1. *In situ* hybridization analysis of human β -globin transcription in E10.5 blood and E13.5 liver cells of EKLF knock-out mice

Genotype	Time point	Percentage of cells with human β -globin nuclear signal	Percentage of cells with α -globin nuclear signals
μ LCR/ β -globin, $EKLF^{+/+}$	E10.5	98	98
μ LCR/ β -globin, $EKLF^{-/-}$	E10.5	56	94
μ LCR/ β -globin, $EKLF^{+/+}$	E13.5	95	98
μ LCR/ β -globin, $EKLF^{-/-}$	E13.5	<1	96

embryos (Bonnerot and Nicolas, 1993). At E10.5, erythroid expression of the μ LCR-*lacZ* transgene is easily detectable in $EKLF^{-/-}$ mice (Figure 2B). X-gal staining of erythroid cells is greatly reduced in the (multi-copy) 5'HS3-*lacZ* line (not shown) and undetectable in the (single copy) core 5'HS3-*lacZ* line (Figure 2C); this is particularly evident when the yolk sacs are stained (compare with Figure 2D and E). Note that the position effect, i.e. *lacZ* expression outside the erythroid system (Tewari *et al.*,

1996), is unaffected in the core 5'HS3-*lacZ*/ $EKLF^{-/-}$ embryos (Figure 2C). To demonstrate that the observed effects on *lacZ* activity are due to reduced transcription of the transgenes, we performed *in situ* hybridizations to determine the percentage of cells actively transcribing the *lacZ* reporter gene (Wijgerde *et al.*, 1995; Tewari *et al.*, 1996). These experiments are summarized in Table II and show that there is a small reduction (from 14 to 10%) in the number of cells with *lacZ* transcription spots in μ LCR-

Fig. 2. Activation of μ LCR- and 5'HS3-lacZ reporter genes in EKLf knock-out mice. (A) Schematic drawing of the transgene constructs. The multi-copy μ LCR-lacZ and 5'HS3-lacZ transgenic lines have been described (lines 'b' in Tewari *et al.*, 1996), the core 5'HS3-lacZ line carries a single copy of the transgene. (B) Embryos were dissected at E10.5, fixed and stained for β -galactosidase activity as described previously (Bonnerot and Nicolas, 1993; Tewari *et al.*, 1996). Shown are a μ LCR-lacZ/EKLf^{+/+} (left) and a μ LCR-lacZ/EKLf^{-/-} (right) embryo. (C) As (B), except that core 5'HS3-lacZ/EKLf^{+/+} (left) and core 5'HS3-lacZ/EKLf^{-/-} (right) embryos are shown. Arrows indicate the presence of embryonic blood in the heart which does not stain blue in the core 5'HS3-lacZ/EKLf^{-/-} embryo. Note that the position effect of the core 5'HS3-lacZ line is unaffected by the EKLf knock-out (see text). (D) X-gal stained yolk sacs of E10.5 core 5'HS3-lacZ/EKLf^{+/+} and (E) core 5'HS3-lacZ/EKLf^{-/-} embryos, showing the absence of β -galactosidase activity in circulating EKLf^{-/-} primitive cells; arrowheads indicate blood-filled arteries.

lacZ/EKLf^{-/-} transgenics. In 5'HS3-lacZ/EKLf^{-/-} and core 5'HS3/EKLf^{-/-} mice, there is a >10-fold reduction in the number of cells with lacZ transcription spots; a >10-fold reduction was also found in E13.5 fetal liver cells of core 5'HS3/EKLf^{-/-} mice, as expected. Together, these data imply that EKLf is not essential for transcriptional activation by the μ LCR, in agreement with observations on the μ LCR- β -globin transgene (this paper) and the complete human β -globin locus (Perkins *et al.*, 1996; Wijgerde *et al.*, 1996). However, the activity of 5'HS3 and the core of 5'HS3 is dependent on the presence of EKLf. Furthermore, our data show unambiguously that EKLf is a functional transcriptional activator in primitive cells. This activity of EKLf has previously gone unnoticed (Nuez *et al.*, 1995; Perkins *et al.*, 1995; Wijgerde *et al.*, 1996).

Increased EKLf levels expedite the switch from γ - to β -globin expression

It has been proposed that globin switching is mediated through a gradual change in the transcription factor environment (Grosveld *et al.*, 1993), and previous experiments carried out with transgenic mice harbouring the complete human β -globin locus showed that the switch

Table II. *In situ* hybridization analysis of lacZ transcription in E10.5 blood of EKLf knock-out mice

Genotype	Percentage of cells with lacZ nuclear signal	Percentage of cells with α -globin nuclear signals
μ LCR-lacZ, EKLf ^{+/+}	14	95
μ LCR-lacZ, EKLf ^{-/-}	10	92
5'HS3-lacZ, EKLf ^{+/+}	15	97
5'HS3-lacZ, EKLf ^{-/-}	1	97
core5'HS3-lacZ, EKLf ^{+/+}	19	98
core5'HS3-lacZ, EKLf ^{-/-}	<1	92

from γ - to β -globin expression, which occurs in the fetal liver between E12 and E14, is delayed in EKLf^{+/+} mice (Wijgerde *et al.*, 1996). This suggests that EKLf is present at critical levels in switching cells, thus we asked if increased EKLf levels would also influence switching. To investigate this, we cloned a cDNA encoding the mouse EKLf protein in the expression vector pEV3 (Needham *et al.*, 1992). This vector contains the μ LCR, the first 400 bp of the β -globin promoter, a modified β -globin gene and the 3' flanking region of the β -globin gene (Figure 3A). Two transgenic mouse lines (EKLf-

Fig. 3. Expression of EKLf under the control of the β -globin promoter and LCR. (A) The cDNA encoding EKLf was cloned in the expression vector pEV3 (Needham *et al.*, 1992). The LCR and a modified version of the β -globin gene, used to drive expression of EKLf cDNA in erythroid cells, are shown. Restriction enzyme sites used to isolate the microinjection fragment for transgenesis are indicated. (B) Western blot analysis of EKLf expression. Ten μ g of protein, isolated from E13.5 fetal livers with the indicated genotype (wt, wild-type; tg, pEV-EKLf transgenic), were fractionated on a 12.5% PAA-SDS gel and blotted onto a PVDF membrane. The blot was probed with a rabbit anti-EKLf polyclonal antibody (top). As a loading control, the blot was reprobed with a mouse monoclonal antibody recognizing the 62 kDa subunit of TF-IIIH. (C) The effect of increased EKLf levels on γ - and β -globin gene expression. Mice carrying the complete 70 kb human β -globin locus were crossed with the EKLf-pEV transgenics. Fetuses were dissected at the stages indicated, genotyped, and the expression of human γ - and β -globin mRNA was determined with quantitative S1-nuclease protection analysis (Wijgerde *et al.*, 1996). The reduction in the γ : β ratio in the presence of the pEV-EKLf transgene was consistently found in three independent litters for each time point (data not shown). The probes were labelled to approximately equal specific activities.

pEV) were generated with this construct. Western blot analysis of E13.5 fetal liver protein shows modest over-expression (2- to 3-fold) of EKLf in one of these lines (Figure 3B). This line was crossed with mice containing the human β -globin locus (Strouboulis *et al.*, 1992) to analyse the expression of γ - and β -globin mRNA in E12.5 and E13.5 fetal liver. The results of the S1 nuclease analysis are shown in Figure 3C. The level of endogenous mouse α -globin mRNA was used to control for the amount of RNA used in each sample. Quantitation of these data reveals that the ratio of γ / β -globin expression decreases earlier in mice carrying the EKLf-pEV transgene, i.e. with a higher EKLf level. Thus, we conclude that the

rate of γ - to β -globin gene switching correlates with the level of EKLf (Wijgerde *et al.*, 1996).

The EKLf transgene rescues the lethal phenotype of the EKLf null mutation

The EKLf-pEV mice should express transgene-derived EKLf in primitive and definitive cells (Hanscombe *et al.*, 1991). Thus, this transgene might rescue the lethality of the EKLf null mutation. To test this, we bred the EKLf-pEV transgene into the EKLf^{-/-} background. In the F2 generation, we obtained mice with an EKLf-pEV/EKLf^{-/-} genotype with both transgenic lines at the expected Mendelian ratio (data not shown). As could be expected,

Table III. Haematological parameters of adult pEV-EKLf rescue mice

	EKLf +/+ (n = 7)	EKLf +/- (n = 7)	EKLf-/- pEV-EKLf (n = 7)	EKLf+/+ pEV-EKLf (n = 6)
RBC ($\times 10^{12}$)	8.8 $\pm$ 0.5	8.3 $\pm$ 0.5	6.5 ^a $\pm$ 0.8	8.9 $\pm$ 0.6
HGB (mmol/l)	8.2 $\pm$ 0.3	7.6 ^b $\pm$ 0.5	6.4 ^a $\pm$ 0.8	8.0 $\pm$ 0.5
HCT (l/l)	0.51 $\pm$ 0.02	0.49 $\pm$ 0.03	0.41 ^a $\pm$ 0.05	0.51 $\pm$ 0.03
MCV (fl)	58.4 $\pm$ 2.4	59.6 $\pm$ 1.5	63.7 ^a $\pm$ 3.4	57.4 $\pm$ 1.5
PLT ($\times 10^9$)	1360 $\pm$ 63	1391 $\pm$ 138	1213 ^b $\pm$ 139	963 ^a $\pm$ 50

RBC, red blood cells; HGB, haemoglobin; HCT, haematocrit; MCV, mean cell volume; PLT, platelets.

^aindicates values that are very significantly different from the wild-type ($p < 0.004$) using a Student's or Welch unpaired *t*-test. Data are given $\pm$ SD.

^bindicates values that are significantly different from the wild-type ($0.04 > p > 0.004$).

the EKLf-pEV transgene also rescues expression in the primitive cells of the 5'HS3-*lacZ* mice discussed above (data not shown).

Haematological analysis of adult mice shows that EKLf-pEV/EKLf^{-/-} mice have lower red blood cell counts, haemoglobin content and haematocrit in comparison with wild-type mice, while the mean cell volume of the erythrocytes is increased (Table III). Thus, erythropoiesis is still slightly impeded in the rescue mice, which might be caused by inadequate expression of transgene-derived EKLf. In addition, we observe that platelet counts are lower in EKLf-pEV/EKLf^{-/-}, and even further reduced in EKLf-pEV/EKLf^{+/+} mice. Finally, in agreement with the analysis of fetal erythropoiesis (Nuez *et al.*, 1995; Perkins *et al.*, 1995), the EKLf^{+/-} mice have haematological values that are close to those found for EKLf^{+/+} mice, although the slightly reduced haemoglobin content might indicate a very mild phenotype.

In conclusion, we show that the expression of transgene-derived EKLf rescues the lethal phenotype of the EKLf null mutation. This formally proves that the phenotype of the EKLf^{-/-} fetuses was indeed the result of the mutation in the EKLf gene, and not some other defect in the ES cells or an unknown other gene that might be present at the EKLf locus.

Discussion

Expression of the β -globin gene is not completely dependent on EKLf in primitive cells

EKLf is essential for high level expression of the adult β -globin genes, and EKLf^{-/-} mice die of anaemia at E14–15 due to defective erythropoiesis caused by severely reduced β -globin levels (Nuez *et al.*, 1995; Perkins *et al.*, 1995). Transgenic mice with just a β -globin gene driven by the μ LCR express the transgene prematurely in primitive cells (Hanscombe *et al.*, 1991); we have used a single copy transgenic line with such a construct (Ellis *et al.*, 1996) to assess the potential role of EKLf in embryonic β -globin expression. Surprisingly, there is only a modest reduction in human β -globin mRNA levels in E10.5 EKLf^{-/-} embryos. This reduction is reflected in the number of cells actively transcribing the transgene, as analysed by primary transcript *in situ* hybridization (Wijgerde *et al.*, 1995). In EKLf^{+/+} cells, transcription foci of the transgene are detected in the nuclei of >95% of the erythroid cells, and in EKLf^{-/-} cells in ~55% of the nuclei. This indicates that the β -globin gene is transcribed only part of the time in $-/-$ primitive cells, which could be attributed to a less

stable interaction between the LCR and the β -globin promoter (Milot *et al.*, 1996). However, the gene is transcriptionally competent and still transcribed at high levels. This is in sharp contrast with the observations in definitive cells of E13.5 EKLf^{-/-} fetal liver. Primary transcript *in situ* hybridization shows a dramatic reduction in the number of cells actively transcribing the β -globin gene. In agreement with data on the expression of the β -globin gene in the intact globin locus (Wijgerde *et al.*, 1996), fewer than 1% of the cells contain a nuclear transcription focus of the transgene. In control littermates, a transcription signal is detectable in >95% of the cells. Thus, expression of the β -globin gene is strictly dependent on the presence of EKLf in definitive cells. However, the expression in primitive cells shows that the β -globin gene is not intrinsically dependent on EKLf. Rather, this dependency is acquired in definitive cells through an as yet unidentified mechanism. A more restrictive chromatin environment in definitive cells, as has been proposed to explain the embryonic expression pattern of many μ LCR-*lacZ* transgenes, could be such a mechanism (Tewari *et al.*, 1996). Consequently, the autonomous silencing of the ϵ - and γ -globin genes (Grosfeld *et al.*, 1993) might at least in part be an intrinsic property of these genes mediated by repressive chromatin interactions.

EKLf is required for the function of 5'HS3 and is a transcriptional activator in primitive erythropoiesis

The promoter of the human β -globin gene contains a canonical binding site for EKLf (Feng *et al.*, 1994). Thus, it is likely that direct binding of EKLf to this element is mandatory for high level expression of the gene in definitive cells (Perkins *et al.*, 1996; Wijgerde *et al.*, 1996). In addition, EKLf binding sites are found in the hypersensitive sites of the LCR (Grosfeld *et al.*, 1993). The best example is provided by 5'HS3, the only element of the LCR that can reproducibly direct expression of single copy globin transgenes in mice and, therefore, has been postulated to provide a dominant chromatin opening activity (Ellis *et al.*, 1996). The 225 bp core fragment of 5'HS3 contains six potential EKLf binding sites (Philipsen *et al.*, 1990). Intactness of these binding sites is essential for the activity of 5'HS3 (Philipsen *et al.*, 1993). Due to the overlapping binding specificity of proteins present in erythroid nuclei, most notably those belonging to the Sp1 family of transcription factors, it is not known which proteins bind to these sequences *in vivo* (Philipsen *et al.*, 1993). To separate the LCR binding sites from those of the promoter, we used mice with a *lacZ* reporter gene

and a non-erythroid minimal promoter linked to LCR derivatives (Tewari *et al.*, 1996) and analysed reporter gene expression in EKLF null mutants. Consistent with the analyses of the β -globin loci (Perkins *et al.*, 1996; Wijgerde *et al.*, 1996), the μ LCR-*lacZ* construct is still active in EKLF^{-/-} embryos. In contrast, expression of the 5'HS3-*lacZ* and core 5'HS3-*lacZ* transgenes is greatly reduced in E10.5 blood, showing that EKLF is required for transcriptional activation by 5'HS3. These results suggest that EKLF binds directly to the core fragment of 5'HS3. Our observations are consistent with the reduced hypersensitivity of 5'HS3 in EKLF^{-/-} fetal livers (Wijgerde *et al.*, 1996) and the phenotype of the 5'HS3 deletion in mice (Hug *et al.*, 1996). This deletion has a very small effect on the expression of the linked embryonic $\epsilon\gamma$ and β h1 genes, while adult β -globin expression is reduced by 30%.

The results with the *lacZ* transgenics show that EKLF is a functional transcriptional activator in primitive cells, in addition to its previously described activity in definitive cells (Nuez *et al.*, 1995; Perkins *et al.*, 1995; Wijgerde *et al.*, 1996). Thus, EKLF may activate specific genes in the primitive lineage, but reduced expression of these genes in EKLF^{-/-} embryos would not result in an obvious phenotype. Experiments aimed at the identification of such EKLF target genes in the primitive and definitive lineage are in progress.

The level of EKLF influences globin gene switching

We have assessed the effect of increased EKLF levels on γ - to β -globin switching in transgenic mice with the complete human β -globin locus (Strouboulis *et al.*, 1992). Globin switching is a gradual process in which the cells change from γ - to β expression over a period of ~2 days (Wijgerde *et al.*, 1995). It has been proposed that switching is effectuated by subtle changes in transcription factor environment (Grosveld *et al.*, 1993). In accord with this notion, our data show that higher EKLF levels result in an earlier switch from γ - to β -globin expression in the fetal liver. Thus, the timing of switching is influenced by EKLF levels, and higher than normal EKLF levels expedite the switch. These data are compatible with the prevailing model that the LCR acts as a holocomplex activating only one gene at a time (Wijgerde *et al.*, 1995). EKLF might increase the stability of interactions between the LCR and the β -globin promoter. Hence, we propose that higher EKLF levels will reduce the time the LCR spends activating the γ -globin genes, thus resulting in an earlier switch (Wijgerde *et al.*, 1996). After switching has completed, increased EKLF levels no longer affect β -globin gene expression, presumably because EKLF is present at saturating levels in adult erythroid cells. This is consistent with the observation that the mouse and human β -globin genes are expressed at wild-type levels in EKLF^{+/-} mice (Wijgerde *et al.*, 1996).

The EKLF-pEV transgene rescues the lethal phenotype of EKLF^{-/-} mice

We show that the lethal phenotype of EKLF^{-/-} mice can be rescued by expressing EKLF under the control of the β -globin LCR. The haematological analysis shows that, compared with wild-type mice, adult EKLF rescue mice have lower red blood cell numbers, haemoglobin concen-

tration and haematocrit, and a slightly increased mean cell volume. Thus, a subtle phenotype remains in these rescue mice which indicates that appropriate developmental expression of EKLF is important for normal erythropoiesis. This notion is emphasized by the observation that pEV-EKLF/EKLF^{+/-} mice have significantly reduced numbers of platelets, suggesting that EKLF may affect the balance between the megakaryocytic and erythroid lineages. Hence, there might be an unexpected link between EKLF and megakaryopoiesis, as has recently been described for another erythroid-specific transcription factor, GATA-1 (Shivdasani *et al.*, 1997).

The rescue experiment sets a platform for the analysis of EKLF protein domains required for high level β -globin expression. EKLF transactivation domains have been characterized in cell culture assays, and a cellular factor interacting with EKLF has been implicated in EKLF-mediated transactivation (Chen and Bieker, 1996). These observations can now be stringently tested through the rescue of EKLF^{-/-} mice. Furthermore, co-immunoprecipitation of EKLF and its interaction partners from primary erythroid cells could be attempted after the introduction of an epitope tag into the EKLF transgene.

Materials and methods

Transgenic mice

The EKLF-pEV construct was generated by inserting the EKLF cDNA into the *Bgl*III site of the pEV3 expression vector (Needham *et al.*, 1992). The microinjection fragment was released by digestion with *Aat*II and *Asp*718, gel purified and used for transgenesis as described (Kollias *et al.*, 1986). The 225 bp core fragment of 5'HS3 (Philipsen *et al.*, 1990) was cloned in a *lacZ* reporter vector (Kothary *et al.*, 1988) and digested with *Hind*III and *Asp*718 to obtain the microinjection fragment. The other transgenic lines used in this study have been described before: μ LCR- β -globin line μ D 14 (Ellis *et al.*, 1996); μ LCR-*lacZ* line b and 5'HS3-*lacZ* line b (Tewari *et al.*, 1996); 70 kb human β -globin locus line 72 (Strouboulis *et al.*, 1992) and EKLF knock-out mice (Nuez *et al.*, 1995).

DNA and RNA analysis

Genomic mouse DNA was prepared from part of the body of the fetuses or tail clips from adult mice. The mice were genotyped by Southern blot analysis (Strouboulis *et al.*, 1992; Nuez *et al.*, 1995; Ellis *et al.*, 1996; Tewari *et al.*, 1996). RNA was isolated from erythroid tissues and globin gene expression was quantitated by S1 nuclease analysis as described (Strouboulis *et al.*, 1992; Wijgerde *et al.*, 1996). Northern blotting was done as described (Talbot *et al.*, 1989). Probes used were the complete EKLF cDNA and a cDNA detecting the erythroid-specific form of aminolevulinate synthase (ALA-S), a kind gift of Dr Peter Curtis (Boston).

Protein analysis

Protein was isolated from E13.5 liver, and 10 μ g was used for Western blot analysis with a rabbit anti-EKLF polyclonal antibody (Southwood *et al.*, 1996), a kind gift of Dr J.J.Bieker (New York). A mouse monoclonal antibody recognizing the 62 kDa subunit of THII-H was used as a loading control; this antibody was generously provided by Drs B.Winkler and G.Weeda (Rotterdam).

LacZ staining

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 and 0.5% glutaraldehyde in PBS for 30 min. They were then rinsed in embryo buffer (PBS, 0.02% NP40, 0.01% deoxycholate, 2 mM MgCl₂) and incubated in staining solution [1 mg/ml X-gal, 5 mM K₃Fe(CN)₆, 5 mM K₄Fe(CN)₆ and 1 mM EGTA in embryo buffer]. After 3–4 h 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.

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. Twenty µl of this cell suspension was fixed onto a poly-lysine coated slide in 4% formaldehyde, 5% acetic acid for 20 min at room temperature. The cells were then washed three times for 10 min in PBS and stored in 70% ethanol at -20°C.

Three probes were utilized for *in situ* hybridization. A nick-translated biotinylated *lacZ* probe was used for the detection of *lacZ* transcription (Tewari *et al.*, 1996). A set of four biotinylated oligonucleotides was used to detect human β -globin transcription (Dillon *et al.*, 1997). DIG-labelled mouse α -globin intron-specific oligonucleotides were used to reveal the erythroid cells (Tewari *et al.*, 1996). Hybridizations were done as described (Wijgerde *et al.*, 1995; Tewari *et al.*, 1996). The *lacZ* hybridization mix contained 50% formamide and 10% dextran sulfate. For antibody detection, the Tyramid amplification system (TSA) of Dupont was used (Raap *et al.*, 1995). For quantitation, ~300 cells were scored for each slide. The reproducibility of these data fell within a 10% error margin.

Haematological analysis

Adult mice (3–5 months of age) were bled by orbital bleeding, and blood samples were analysed on an F800 microcell counter. Statistical analysis was done by unpaired two-sided *t*-testing.

Acknowledgements

We thank Lien Braam for animal husbandry. R.T. and M.W. were supported by an N.W.O. (The Netherlands) grant to F.G.

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Received November 17, 1997; revised January 5, 1998;
accepted February 2, 1998