The role of EKLF in human β-globin gene competition

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We have investigated the role of erythroid Kruppel-like factor (EKLF) in expression of the human β-globin genes in compound EKLF knockout/human β-locus transgenic mice. EKLF affects only the adult mouse β-globin genes in homozygous knockout mice; heterozygous mice are unaffected. Here we show that EKLF knockout mice express the human ϵ and γ-globin genes normally in embryonic red cells. However, fetal liver erythropoiesis, which is marked by a period of γ- and β-gene competition in which the genes are alternately transcribed, exhibits an altered ratio of γ- to β-gene transcription. EKLF heterozygous fetal livers display a decrease in the number of transcriptionally active β genes with a reciprocal increase in the number of transcriptionally active γ genes. β-gene transcription is absent in homozygous knockout fetuses with coincident changes in chromatin structure at the β promoter. There is a further increase in the number of transcriptionally active γ genes and accompanying γ gene promoter chromatin alterations. These results indicate that EKLF plays a major role in γ- and β-gene competition and suggest that EKLF is important in stabilizing the interaction between the Locus Control Region and the β-globin gene. In addition, these findings provide further evidence that developmental modulation of globin gene expression within individual cells is accomplished by altering the frequency and/or duration of transcriptional periods of a gene rather than changing the rate of transcription.

[*Key Words*: Erythroid Kruppel-like factor; locus control region; gene competition; β-globin; transcription]

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The human β-globin locus contains five functional erythroid-specific genes arranged in the order of their developmental expression (5′-ϵ–$^{G}\gamma$–$^{A}\gamma$–δ–β-3′) (for review, see Collins and Weissman 1984). The sequences required for correct developmental globin gene regulation have been the target of intensive studies in transgenic mice. Early studies in which individual globin genes were introduced were hampered by lack of expression or low non-copy number-dependent expression, suggesting that expression was dependent on the position of integration in the mouse genome. Although made difficult by these position effects the results suggested that the individual genes contained information neccessary for proper developmental regulation. The inclusion in globin gene constructs of the locus control region (LCR), which consists of 5 DNAse I hypersensitive sites (HS:1–5) located upstream of the cluster, permitted reliable position-independent, copy number-dependent expression at levels equivalent to the endogenous mouse globin genes (Grosveld et al. 1987). Subsequent experiments with individual globin genes linked to the full LCR have shown that the ϵ- and γ-globin genes are regulated autonomously (Raich et al. 1990; Dillon and Grosveld 1991). The ϵ gene is restricted to embryonic red cells and the γ gene is expressed in both embryonic- and fetal-derived red cells until it is silenced autonomously around day 16. The adult β gene, on the other hand, is expressed aberrantly at early stages when linked to the LCR and is regulated properly only when another gene is placed in *cis* between it and the LCR, suggesting that the β gene is regulated competitively (Hanscombe et al. 1991). Transgene experiments in which γ and β gene order with respect to the LCR has been varied have demonstrated a developmental expression pattern altering effect (Hanscombe et al. 1991; Peterson and Stamatoyannopoulos 1993; Dillon et al. 1995; N. Dillon and F. Grosveld, in prep.). When a marked β gene is placed in the ϵ position in the context of the full locus it is expressed throughout development as expected, but, remarkably, it also completely suppresses the fetal and adult expression from the downstream γ and β genes in the locus (Dillon et al. 1995; N. Dillon and F. Grosveld, in prep.).

These data have led us to propose a model for LCR-driven β-globin gene expression in which the individual HS of the LCR act together (or form a holocomplex) and interact directly with an individual globin gene. In this model the complete LCR plays a pivotal role in gene competition. This is supported by the fact that no individual HS of the LCR provides full expression to a linked globin gene (Fraser et al. 1990, 1993), and that all sites are required in the context of the full locus for high-level,

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position-independent expression (Milot et al. 1996). The competitive advantage of an individual gene is achieved through proximity to the LCR and the specific *trans*-acting factor environment of a particular developmental stage. We propose that these parameters are the basic determinants that control the frequency and duration of direct LCR/gene interactions, which in turn control transcription. We have shown through in situ primary transcript detection that coexpression of multiple genes from a single locus involves alternate rather than cotranscription (Wijgerde et al. 1995). These results indicate that dynamic LCR–gene interactions are the mechanisms of gene competition and coexpression. Obviously, an important question is, which proteins mediate complex formation and stability?

Erythroid Kruppel-like factor (EKLF) is a protein with three zinc fingers that show homology to members of the Kruppel-like family of nuclear proteins such as TFIIIA and Sp1 (Miller and Bieker 1993). EKLF is largely restricted to erythroid cells and binds CACC-box sequences (CCACACCCT) (Donze et al. 1995) frequently found in erythroid specific gene promoters and duplicated in the adult mouse and human β-globin gene promoters. Naturally occurring single base mutations in the β-globin CACC box result in mild to severe reductions in β-globin gene expression in human thalassemic patients (Orkin et al. 1982, 1984; Kulozik et al. 1991). Similarly, constructs bearing these base substitutions show decreased *trans*-activation of linked reporter genes (Feng et al. 1994). EKLF null mice express endogenous embryonic β-like globins and appear normal during embryonic development (Nuez et al. 1995; Perkins et al. 1995). As the site of erythropoiesis changes to the fetal liver (day 11.5 onward), $EKLF^{-/-}$ animals fail to express the mouse adult β-globin genes and die in utero around day 15. $EKLF^{+/-}$ mice appear normal in terms of β-major and β-minor gene expression (Nuez et al. 1995, Perkins et al. 1995). Because both genes contain CACC boxes in their promoters and are dependent on EKLF for expression (Nuez et al. 1995; Perkins et al. 1995), they are not informative in studies on gene competition (Shehee et al. 1993; T. Trimborn and P. Fraser, unpubl.).

Here we demonstrate the role of EKLF in human β-globin gene switching through analyses of transgenic animals homozygous for a single copy of the entire human β-globin locus in an EKLF heterozygous or homozygous null background. The results show that the absence of EKLF leads to a complete lack of β-gene expression with a concomitant increase in γ gene expression in fetal liver-derived erythroid cells, whereas a decrease in EKLF in heterozygous mice leads to a different ratio of γ to β expression during the switching period. These results indicate that EKLF plays a major role in γ- and β-gene competition and suggest that EKLF is important in the LCR/β-gene interaction.

Results

Embryonic erythropoiesis

Transgenic mouse line 72 (Strouboulis et al. 1992), which carries a single copy of the complete human β-globin locus, was crossed with a targetted EKLF mutant line (Nuez et al. 1995). Offspring were bred to produce three genotypes for analysis: $Hu\beta^{+/+}/EKLF^{+/+}$ (line 72 homozygous/EKLF wild type), $Hu\beta^{+/+}/EKLF^{+/-}$ (line 72 homozygous/EKLF heterozygous knockout), and $Hu\beta^{+/+}/EKLF^{-/-}$ (line 72 homozygous/EKLF homozygous knockout). Phenotypic analysis of embryonic stage mice suggests that development proceeds normally as EKLF null mutant embryos are indistinguishable from heterozygous or wild-type littermates. S1 nuclease protection analysis of day 10 and 11 blood RNA samples showed that human ε- and γ-globin mRNA expression levels are unchanged in EKLF mutant embryos (Fig. 1A and Fig. 2A) as compared with the unaffected mouse α-globin genes (Nuez et al. 1995; Perkins et al. 1995). Primary transcript in situ hybridization analysis of day 10 embryonic blood cells showed no differences in the number and distribution of active transcriptional foci for human ε and γ globin (Wijgerde et al. 1995) among the three different genotypes (not shown). These results indicate that embryonic expression of the human ε- and γ-globin genes is not dependent on EKLF.

Human globin gene expression in the fetal liver

Around day 11 post conception (p.c.) the fetal liver becomes the major site of erythropoiesis. Expression of human ε globin is completely silenced in the fetal liver, whereas γ-gene expression persists in competition with the β gene (Strouboulis et al. 1992; Wijgerde et al. 1995). $EKLF^{+/-}$ mice develop normally in contrast to $EKLF^{-/-}$ mice, which appear abnormal in terms of hemoglobinization as early as 12.5 days and severely anemic by day 14.5 (Nuez et al. 1995; Perkins et al. 1995). In contrast, $Hu\beta^{+/+}/EKLF^{-/-}$ mice still have hemoglobinized peripheral blood and a relatively red fetal liver as late as 15.5 days (not shown). This result suggests that human β-like chains are heterotetramerizing with mouse alpha chains to form hemoglobin in the fetal liver cells.

S1 protection assays performed on total fetal liver RNA showed dramatic differences in the steady-state globin mRNA levels (Figs. 1B and 2B). The normal pattern of γ- and β-gene expression (Strouboulous et al. 1992) was disrupted in heterozygous EKLF knockout fetuses. Human β-gene expression was nearly halved in the early fetal liver cells (11–15 days) when compared with EKLF wild-type fetuses. The decrease in human β-gene expression was accompanied by an increase in γ-gene expression over the same period. This decrease in human β expression is interesting in light of the fact that the endogenous β-major and β-minor genes are unaffected in EKLF heterozygotes (Fig. 1B; Nuez et al. 1995; Perkins et al. 1995). In $Hu\beta^{+/+}/EKLF^{-/-}$ fetuses human β-gene expression is undetectable and γ-gene expression increases by as much as three or fivefold compared with $Hu\beta^{+/+}/EKLF^{+/-}$ and $Hu\beta^{+/+}/EKLF^{+/+}$ fetuses, respectively. The null mutants also showed a

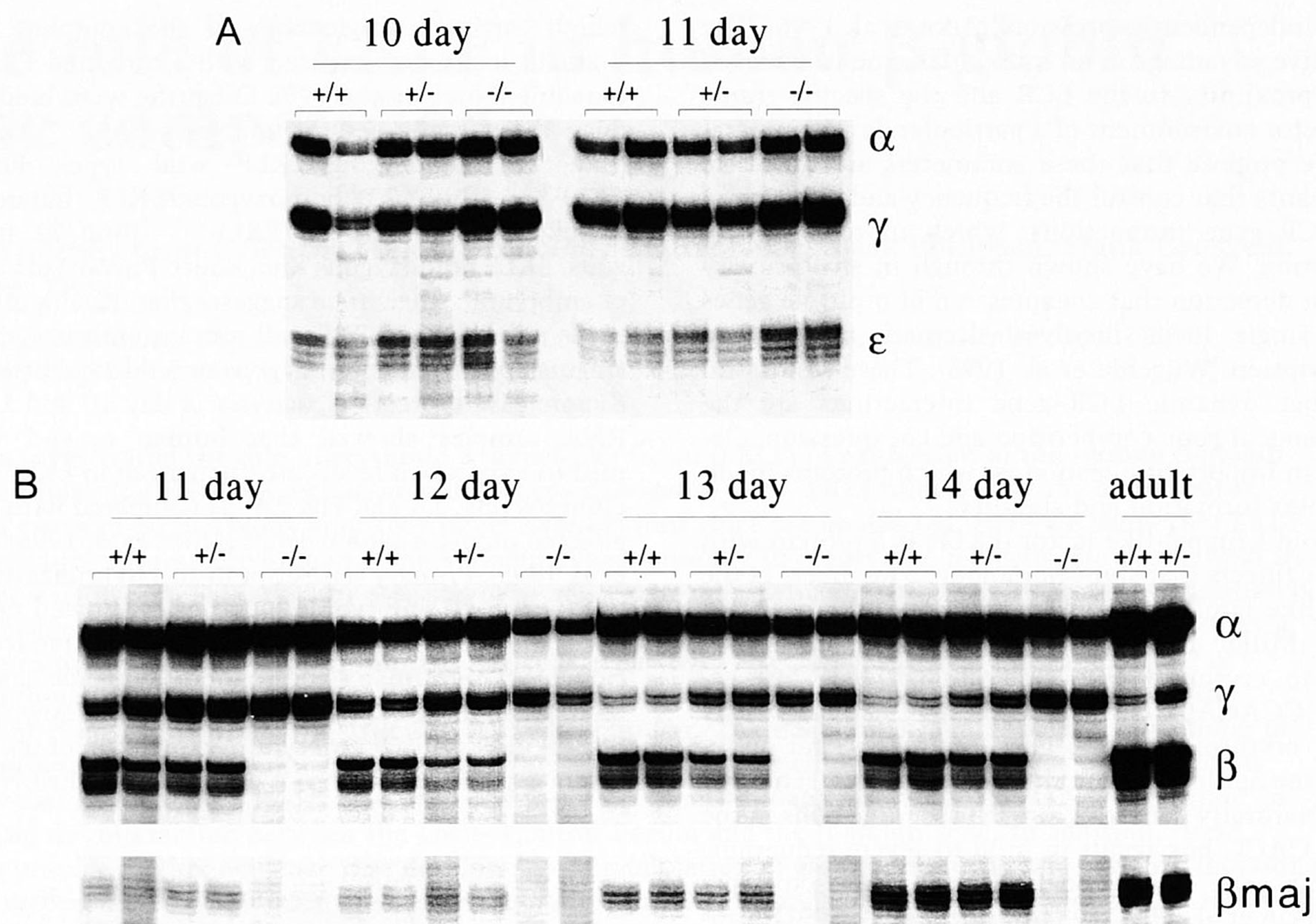

Figure 1. S1 nuclease protection assays. Probes for human ϵ, γ and β globin and mouse α and β-major globin were used as described in Materials and Methods. All mice analyzed were homozygous for a single copy of the entire human β-globin locus (line 72, Strouboulis et al. 1992) in either EKLF$^{+/+}$, EKLF$^{+/-}$, or EKLF$^{-/-}$ background as indicated above each lane. (*A*) Total RNA from 10- and 11-day embryos including yolk sac. (*B*) Eleven- to 14-day fetal livers and adult peripheral blood RNA. Protected products are indicated to the *right* of each panel.

dramatic decrease in the level of mouse β-globin mRNA, as expected (Nuez et al. 1995; Perkins et al. 1995).

There are two potential explanations for the observed increase in γ- and decrease in β-mRNA expression. It is possible that the reduced amount of EKLF in the heterozygous knockout animals abates the transcriptional activity of the β genes by reducing polymerase density and, therefore, rate of transcription of all β genes. However, reduced EKLF levels must also have the opposite effect on the transcription rate of individual γ genes to increase expression. EKLF would then be a purported suppressor of γ-gene transcription, or indirectly affect such a gene product, which we cannot rule out. However, it is known that EKLF is present in embryonic red cells where its presence does not silence γ genes. The other possibility, which fits with our previous results on gene competition, would suggest that competition from the γ gene for the LCR is more successful in an EKLF$^{+/-}$ background as a result of decreased competence of the β gene. In this situation the frequency of LCR–γ gene interactions would be increased because of a decrease in the duration (stability) or frequency of LCR–β gene interactions. This would result in a decrease in the absolute number of transcriptionally active β genes within the population and a reciprocal increase in the number of transcriptionally active γ genes. This is supported by the return to near normal levels of human β-globin expression in the adult blood of EKLF$^{+/-}$ mice when the γ genes are silenced. To differentiate between these two possibilities we quantitated the number of transcriptionally active γ- and β-globin genes in the fetal liver.

Primary transcript in situ hybridization

The analysis of steady-state mRNA levels for human γ and β globin during fetal liver erythropoiesis demonstrated concomitant increases in γ-gene expression with decreases in β-gene expression, confirming the reciprocal relationship in the competition between the genes. Our previous results suggested that if a globin gene was transcriptionally active, it was transcribed at the full rate because changes in mRNA levels could be correlated with changes in the number of transcriptionally active genes (Wijgerde et al. 1995; Milot et al. 1996). Fifty percent expression of a gene at the mRNA level did not suggest that each gene was transcribed at half the normal rate, but rather that half the genes were transcribed at the normal rate (Wijgerde et al. 1995; Milot et al. 1996). In other words, each gene was transcribed for half the time. To further investigate gene competition and γ-gene

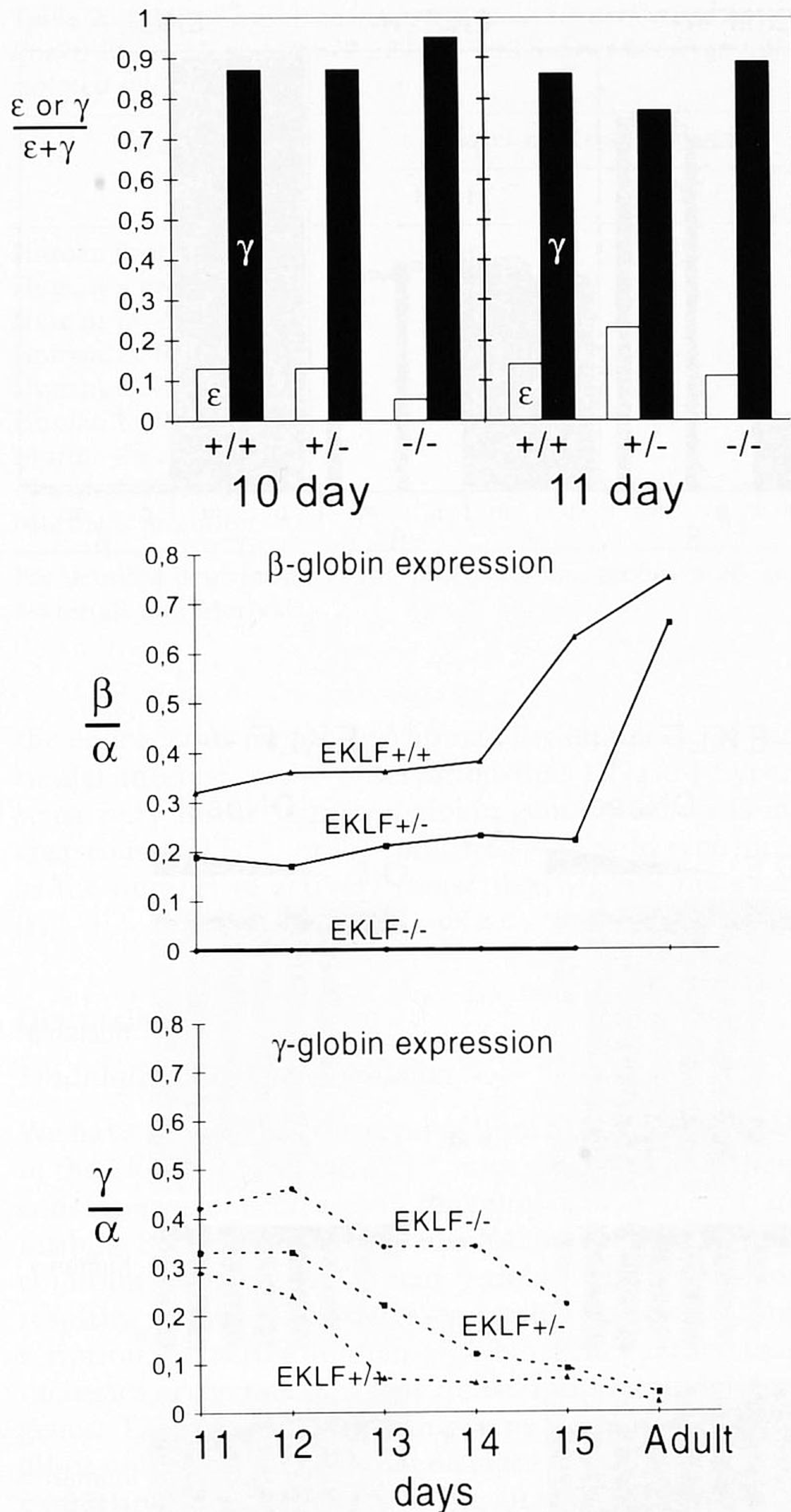

Figure 2. Expression pattern of the human globin transgenes in EKLF null background. Expression levels were quantitated using PhosphorImager analysis of S1 protection assays in Fig. 1, and plotted against the developmental age of the animals. (*A*) Relative expression levels of ϵ- and γ-globin mRNA in day 10 and day 11 whole embryos in $EKLF^{+/+}$, $EKLF^{+/-}$, or $EKLF^{-/-}$ background. (*B*) Expression of human β-globin mRNA during fetal stages of erythropoiesis and in adult peripheral blood as a ratio of mouse α-globin mRNA. (*C*) Expression of human γ-globin mRNA during fetal stages of erythropoiesis and in adult peripheral blood as a ratio of mouse α-globin mRNA.

silencing we analyzed transcription of individual γ- and β-globin genes in single cells using double label primary transcript in situ hybridization (Wijgerde et al. 1995) in 12.5-day fetal liver cells (Fig. 3A and B). The results show that the percentage of transcription foci for the β gene decreases from 80% in the wild type to 52% in $EKLF^{+/-}$ and 0% in $EKLF^{-/-}$ mice (Fig. 4 and Table 1). γ-gene transcription foci increase from 20% in wild type to 48% in $EKLF^{+/-}$ fetuses. Because no human β-globin gene foci were observed in the $EKLF^{-/-}$ fetuses we also compared γ-gene foci with mouse α-globin gene foci in 13.5-day wild type, $EKLF^{+/-}$, and $EKLF^{-/-}$ fetuses (Table 1 and Fig. 3C and D). The results show that in $EKLF^{-/-}$ mice only 41% of the γ genes in the red cells were transcriptionally active, suggesting that many γ genes already may have been irreversibly silenced (Dillon and Grosveld 1991, see Discussion). Correlation of the relative percent of γ and β mRNA in the fetal liver population (S1 analysis) and the number of transcriptionally active γ and β genes (in situ data) indicates that changes in the expression levels of the individual genes are wholly accounted for by changes in the number of transcriptionally active genes (Fig. 4 and Table 1) and not attributable to changes in the rate of transcription. These results confirm that the level of expression of a globin gene is determined by the frequency and duration of its transcription and not by changes in the rate of transcription or polymerase density along a single template. Hence we conclude a gene is either transcribed fully or is silent.

DNaseI hypersensitive site analysis

S1 nuclease protection assays combined with primary transcript in situ hybridization have revealed that the

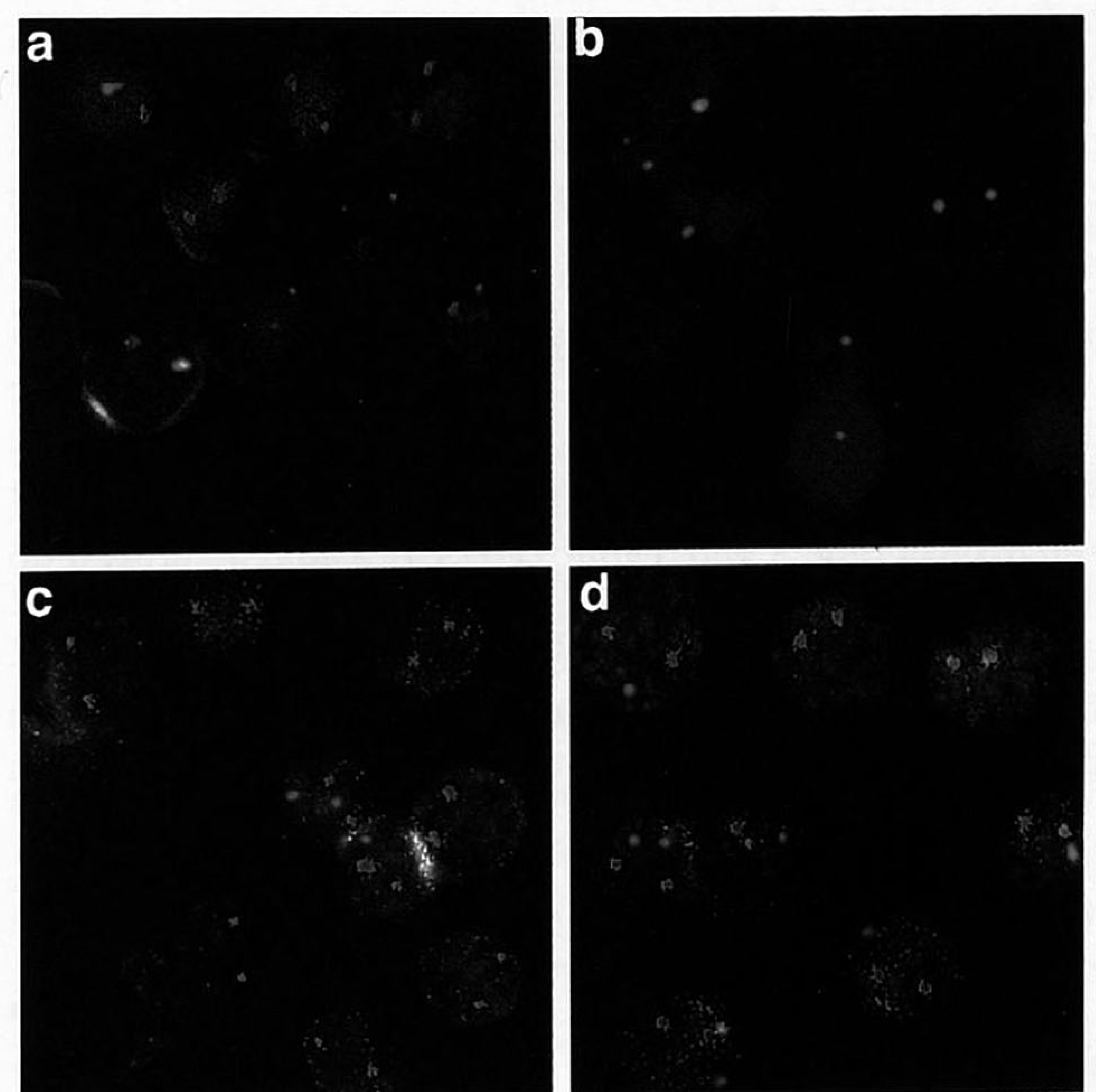

Figure 3. Primary transcript in situ hybridization. EKLF wild-type and knockout 13.5-day mouse fetal liver cells homozygous for the human β-globin locus were hybridized with gene-specific intron probes for (*A*,*B*) human γ (red) and β globin (green) or (*C*,*D*) mouse α globin (green) and human γ globin (red). *A* and *B* were created with a CCD camera. *C* and *D* were created with a laser scanning confocal microscope. Genotypes (*A*) $Hu\beta^{+/+}$ $EKLF^{+/+}$; (*B*) $Hu\beta^{+/+}$ $EKLF^{-/-}$; (*C*) $Hu\beta^{+/+}$ $EKLF^{+/+}$; (*D*) $Hu\beta^{+/+}$ $EKLF^{-/-}$.

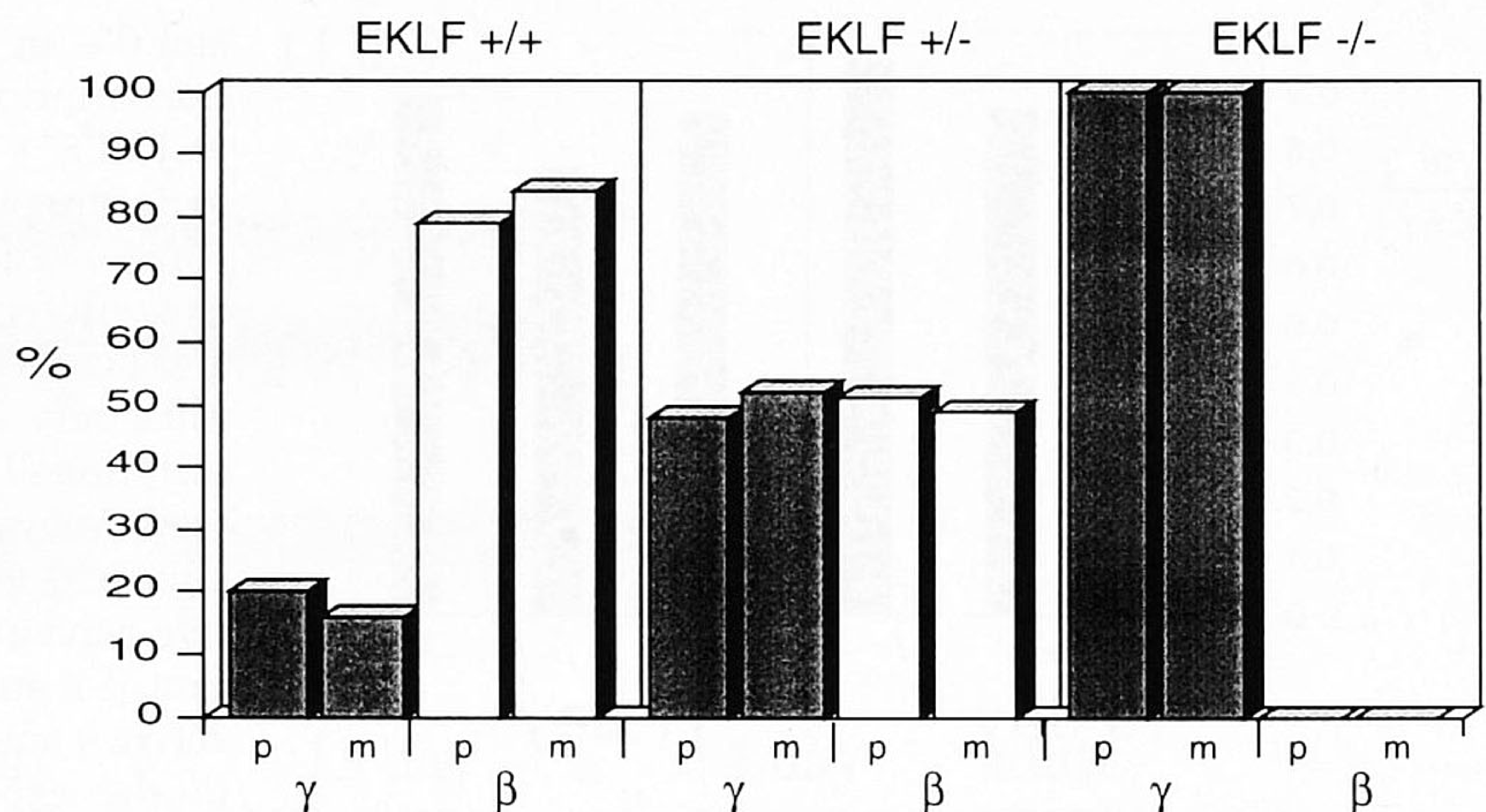

Figure 4. Correlation between transcriptional activity and steady-state mRNA levels. Quantities of steady-state mRNA (γ or β) at 13.5 days postconception from Figs. 1 and 2 are represented as a percentage of total human RNA (γ or β/γ + β). The percentages of transcriptionally active γ and β genes were calculated from the primary transcript in situ data (Table 1; 13.5 days) by dividing the number of transcriptionally active γ or β genes by the total number of transcriptionally active γ and β genes (γ or β/γ + β). Loci with double signals were equally divided between γ and β categories. (p) Primary transcript foci; (m) mRNA levels. Values for EKLF$^{+/+}$, EKLF$^{+/-}$, and EKLF$^{-/-}$ are shown as indicated.

decrease in the steady-state level of human β-globin mRNA is a result of a decrease in the number of actively transcribing β genes in the EKLF$^{+/-}$ and EKLF$^{-/-}$ mice resulting in a concomitant increase in the number of transcriptionally active human γ-globin genes. Globin gene transcription normally correlates with the presence of DNase I hypersensitive sites at the promoter and in the LCR (Tuan et al. 1985; Forrester et al. 1987; Grosveld et al. 1987). To determine the effect of reduced EKLF levels on chromatin structure in the human and mouse β-globin loci, we analyzed DNase I hypersensitivity of promoter and LCR HS regions in isolated nuclei from wild-type and EKLF$^{-/-}$ 13.5-day fetal liver cells. As expected, no difference was seen in the hypersensitivity of the unaffected mouse α-globin gene promoters (Fig. 5). In the absence of EKLF most of the globin locus appears normal in terms of DNase I hypersensitivity (Table 2); however, the adult β-globin promoter is resistant to DNase I digestion (Fig. 5). The mouse β-major globin promoter is also resistant to DNase I and the mouse and human LCR HS3 appear to be reduced to half of normal sensitivity (Fig. 5 and Table 2). HS:1, 2, and 4 showed no difference in sensitivity between wild-type and knockout mice (Table 2). These results suggest that EKLF primarily affects chromatin structure at the adult β-globin promoter and to a lesser extent HS3 of the LCR in both

Table 1. *Quantitation of in situ hybridizations*

	γ	β	γ–β	γ/α
EKLF +/+	13	72	15	18
EKLF +/−	37	41	22	33
EKLF −/−	100	0	0	41

The percentages of loci in 13.5-day fetal liver cells having single gene primary trancript signals for γ or β, and those having double signals γ–β are shown for the three genotypes indicated. Greater than 170 loci were counted for each genotype. γ/α represents the number of transcriptionally active γ genes as a percentage of transcriptionally active mouse α-globin genes. Greater than 500 α-globin loci were counted for each genotype.

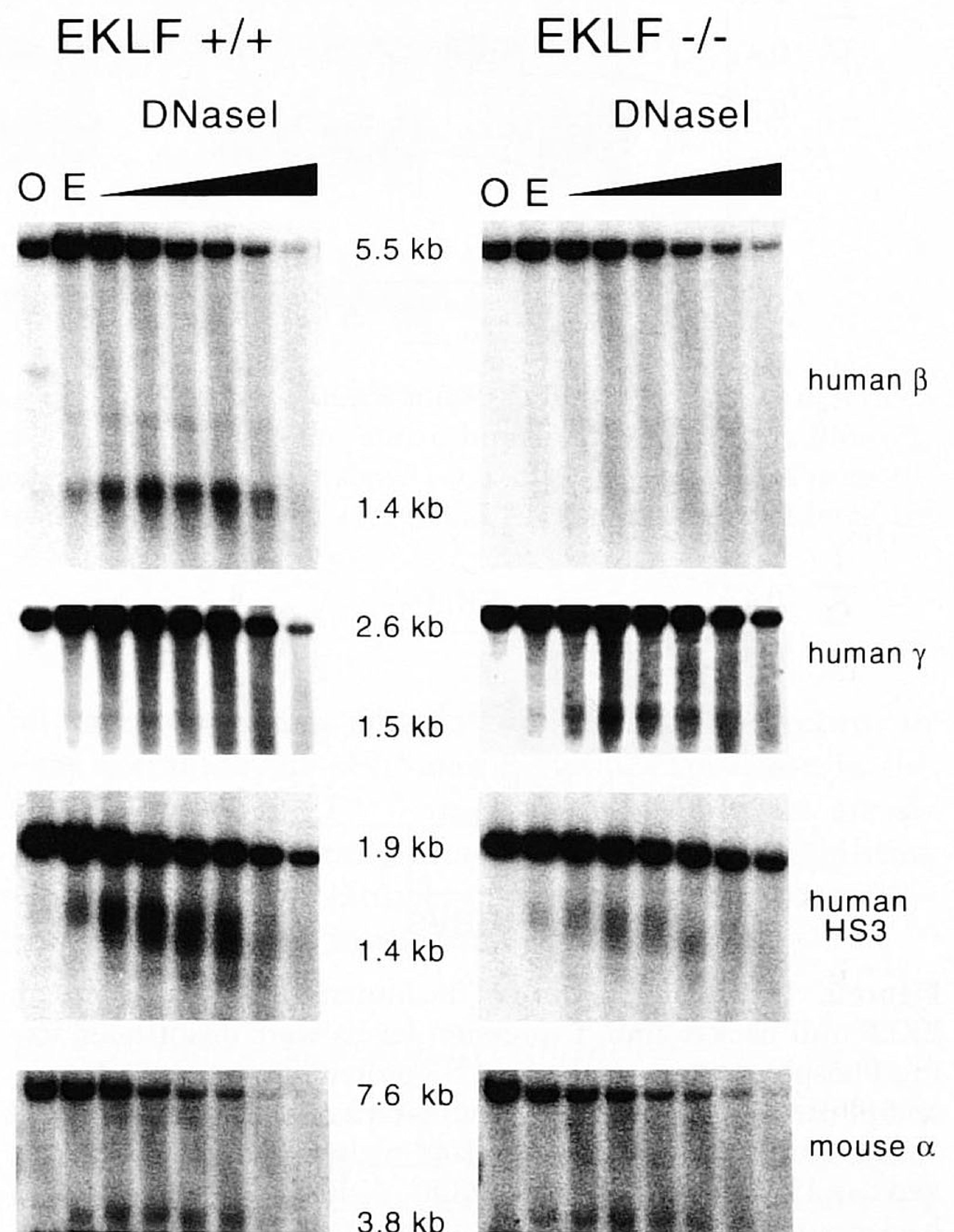

Figure 5. DNase I hypersensitive site analysis of 13.5-day fetal liver cells. Shown are DNase I hypersensitivity analyses in isolated nuclei from fetal livers of mice homozygous for the human β-globin locus in EKLF$^{+/+}$ and EKLF$^{-/-}$ backgrounds. The probes used are described in Materials and Methods and the sizes of parent and hypersensitve bands are indicated. Shown from *top* to *bottom*: Human β-globin promoter (*top* panel, *Eco*RI digest), human γ-globin promoter (*Eco*RI digest), human LCR HS 3 (*Hin*dIII digest), and endogenous mouse α-globin promoter (*bottom* panel, *Sac*I digest). Lanes labeled with O and E indicate samples incubated on ice and 37°C, respectively, without DNase I. The triangled bar above the lanes indicates increasing amounts of DNase I.

Table 2. *DNaseI hypersensitive site analysis performed on line72 EKLF$^{+/+}$ and line72 EKLF$^{-/-}$ 13.5-day fetal liver cell isolated nuclei*

	DNaseI hypersensitive sites	
	EKLF$^{+/+}$	EKLF$^{-/-}$
Human β promoter	+	−
Human γ promoter	−	+
Human LCR HS1	+	+
Human LCR HS2	+	+
Human LCR HS3	+	+/−
Human LCR HS4	+	+
Murine βmaj promoter	+	−
Murine LCR HS3	+	+/−
Murine α promoter	+	+

For detailed description of the procedure and probes used, see Materials and Methods.

the endogenous mouse and human β-globin loci. Of particular interest is the observation that DNase I hypersensitivity of the human γ-globin gene promoter is increased in EKLF$^{-/-}$ mice compared with wild-type mice as the number of actively transcribed γ-genes increases (Fig. 5).

Discussion

Modulation of gene expression

We have shown that the level of human β-globin mRNA in the EKLF$^{+/-}$ and EKLF$^{-/-}$ mice decreases as a direct consequence of decreases in the number of actively transcribing β-globin genes. The results show reciprocal concomitant increases in human γ-globin gene expression resulting solely from increases in the number of transcriptionally active human γ-globin genes rather than increases in the rate of γ-gene transcription on individual genes. These data indicate that gene transcription is an all-or-nothing event and that changes in the level of gene expression reflect changes in the amount of time that a gene is transcribed rather than changes in the rate of transcription of individual genes. These observations support our previous work, which showed that as development proceeds, modulation of the expression level of γ versus β mRNA is determined by the frequency and duration of transcription of the individual genes and not by changes in the rate of transcription from individual promoters (Wijgerde et al. 1995). The genes appeared to be either fully transcribed or off. This concept was independently reinforced by data that showed that novel position effects that cause low-level expression in all cells of a tissue are the result of decreasing the amount of time that a gene is transcriptionally active and not the result of modulation of transcription rate or polymerase density on an individual gene (Milot et al. 1996).

Other groups have proposed a binary mechanism of enhancer action. Robertson et al. (1995) described the silencing of expression of a construct containing an *Escherichia coli lacZ* gene under control of the human α-globin HS-40 element in transgenic mice. It had been shown previously that HS-40 could not maintain high-level copy number-dependent expression of the α-globin gene throughout development as expression levels declined as development proceeded (Sharpe et al. 1992, 1993). Histochemical staining for β-gal showed that the lower expression levels seen late in development correlated with decreasing numbers of *lacZ*-expressing cells, and suggested that a decrease in the rate of transcription from individual promoters did not play a role. A report by Walters et al. (1996) described silencing of an HS:2 β-geo construct in cell culture after recombinase mediated removal of HS:2. They found that after culturing stably transfected clones for several weeks, greater numbers of cells, though variable from line to line, had silenced the β-geo gene when compared with the original HS:2-containing clone. It was suggested that the level of β-gal expression per cell did not change dramatically in those cells that continued to express the β-geo gene, although twofold variation was common. They concluded that enhancers act to prevent silencing of genes and have little if anything to do with the rate of transcription of a gene. In addition, it was concluded that in terms of transcription, a gene was either stably on or off in a particular cell, although in neither report was transcription measured nor was the activation/silencing status of a cell heritable as in position effect variegation (PEV). Both reports show that levels of steady-state mRNA extracted from a population of cells do not always reflect changes in all of the cells. We have observed a similar phenomenon in cases of genuine PEV in which a stable, clonal subpopulation of cells expresses or silences a transgene as a result of juxtaposition to centromeric heterochromatin leading to reductions in mRNA levels in a population of cells (Milot et al. 1996). However, these types of experiments do not allow conclusions to be made about modulation of gene expression in individual expressing cells. We have shown that expression levels of individual genes are modulated within a single cell by altering the periods of active transcription of a gene and not the rate of transcription (Wijgerde et al. 1995; Milot et al. 1996; this report). This result on the dynamics of transcription is fundamentally different from the work on gene silencing mentioned above. Gene transcription appears to be an all-or-nothing event, and modulation of gene expression within a single cell is accomplished through altering the transcriptional periods.

Shifting the balance of globin gene competition

The analyses of human embryonic β-like globin gene expression and transcription in EKLF null mice demonstrate that the ε- and γ-gene promoters are not dependent on EKLF. Similar results were obtained when the mouse embryonic globin genes were analyzed (Nuez et al. 1995; Perkins et al. 1995). Human globin gene expression in the fetal liver of transgenic mice involves a prolonged period of gene competition between the γ- and β-globin genes from day 11.5 to approximately day 16.5 (Strouboulis et al. 1992; Peterson and Stamatoyannopo-

ulos 1993; Wijgerde et al. 1995). During this period it is thought that a gradual change in the transcription factor environment of individual cells leads to changes in the affinity and/or stability of interactions between the LCR and the individual genes, causing a shift in the balance of transcription toward the adult β gene. We have shown previously that expression of multiple globin genes from a single locus involves alternating transcription of individal genes (Wijgerde et al. 1995). We proposed a mechanism whereby the LCR forms an exclusive semistable complex with an individual gene to initiate transcription but is able to flip-flop between genes. The implication of the proposed mechanism is that formation of a stable complex between the LCR and a gene allows loading or initiation of RNA polymerases (and reinitiation) at a fixed rate. The frequency and stability of the complex would therefore determine the level of transcription and disruption of the interaction would prevent further loading of polymerases. Support for the idea that the LCR acts as a functional unit has come from studies that show that all HS of the LCR are required for position-independent and copy number-dependent expression (Milot et al. 1996). Indeed, deletion of individual HS, which one might assume would destabilize the complex, leads to novel postion effects in which transgene expression levels were lower as a result of reduced transcription time (Milot et al. 1996).

Here we show that in EKLF$^{+/-}$ mice the ratio of γ- to β-gene transcription is shifted toward the γ genes when compared with wild-type mice. This shift in the ratio represents an actual increase in the absolute number of actively transcribed γ genes and a decrease in the number of transcribed β genes. Therefore the decreased level of EKLF in heterozygous knockouts leads to a reduction in the amount of time that the LCR spends complexed with the β gene. Because the γ-gene promoter is not directly affected by EKLF we propose that increased γ-gene transcription is the result of increased availability of the LCR as a result of reduction in the time it is occupied by the β gene. Hence LCR–γ gene interactions, though unchanged in stability, are able to form more frequently.

Martin et al. (1996) have described a different model of globin gene regulation. They propose that the LCR acts in concert with elements in the locus to initiate and maintain an active chromatin structure. No contact or interaction is required between the LCR and the genes in the locus for transcriptional activation. Instead, individual genes within the locus have differing probabilities of transcriptional activation depending on gene-local *cis*-elements and the developmentally regulated *trans*-acting factors that bind them. Multiple genes may be transcribed simultaneously from the same locus but 5′ genes may interfere with the transcription of 3′ genes by either lowering the probability or decreasing the rate of transcription. Silencing of the more 5′ genes during development allows activation of the downstream genes. However, the data presented here directly contradicts this model as we have shown that decreases in transcription of a 3′ gene (the β gene) lead to reciprocal increases in a 5′ gene (the γ genes). These results are exactly what one would predict in a case of genuine gene competition, and are consistent with the observations that the switch from γ to β expression is competitive (Hanscombe et al. 1991; Peterson and Stamatoyannopoulos 1993; Dillon et al. 1995; N. Dillon and F. Grosveld, in prep.) and dynamic (Wijgerde et al. 1995; this report).

γ-gene silencing

In the homozygous knockout animals β-gene transcription is not detectable and the percentage of loci actively transcribing the γ-genes increases to 41% of transcriptionally active mouse α-globin loci (Fig. 3 and Table 1). Of interest is the fact that not all γ genes are transcribed in the absence of β-gene transcription. It is known that γ-gene transcription is silenced autonomously during fetal liver development based on expression analysis of a γ-gene linked to the LCR (Dillon and Grosveld 1991). Our observations would predict that the decreasing level of γ-gene expression seen in those experiments is the result of decreasing numbers of transcriptionally active γ-genes (i.e., increasing numbers of silenced loci). One possible explanation is that in the EKLF $^{-/-}$ mice there is a distinct subset of fetal liver cells in which the γ-genes are still capable of transcription (i.e., not silenced). Our results show that the proportion of cells with both loci transcribing the γ genes increases significantly in the double knockout mice compared with wild type and heterozygotes at the expense of cells with only one locus active in γ-gene transcription. However, even in the homozygous knockout the majority of cells that are transcribing γ have only one active locus. It is possible that the other locus appears inactive because the LCR still spends a significant, though reduced amount of time complexed with the β-gene but does not lead to productivity. An alternative explanation is that the γ genes on the other locus are reacting independently to the same factor environment (as observed previously; Wijgerde et al. 1995) and have been silenced thereby making them unavailable for LCR interaction and transcription. Comparison with the results of Dillon et al. (1991) in which γ silencing was measured in the absence of competition from the β-gene suggests that we may be observing a combination of the possibilities described above.

Chromatin structure

The results of DNase I hypersensitive site analysis suggest that the chromatin structure of the human globin LCR remains largely intact in EKLF double knockout mice. HS 1, -2, and -4 of the LCR appear unchanged in the absence of EKLF. The decrease in sensitivity of the promoter of the adult β genes (human and mouse) that is coincident with transcriptional deficiency is quite severe, indicating that EKLF is essential for the proper chromatin structure of the promoter. Hypersensitivity of the γ-gene promoter increases in the EKLF double knockout mice as transcription increases. Our model predicts that this increase is the result of an increase in

the number of γ genes interacting with the LCR. It is not possible to determine with certainty whether hypersensitivity is the result of transcription of the gene or interaction with the LCR, as the two are normally linked. If the LCR is complexed with the β gene in the homozygous knockouts as suggested above, one would then conclude that hypersensitivity is more closely linked to transcription than LCR interaction. EKLF also contributes, to a lesser extent, to the hypersensitivity of HS 3 in the mouse and human LCRs, suggesting that it may directly interact with sequences in HS 3.

Materials and methods

Transgenic mice

Transgenic mice containing a single integrated copy of the human β-globin locus, referred to as Line 72 (Strouboulis et al. 1992), were crossed with EKLF knockout mice (Nuez et al. 1995) to create compound homozygous human β-globin $Hu\beta^{+/+}$/$EKLF^{+/-}$ and $Hu\beta^{+/+}$/$EKLF^{-/-}$ embryos. Embryos were dissected out at various time points and genotyped by Southern blot and PhosporImage analysis.

Preparation of RNA and S1 nuclease protection assay

RNA was extracted from frozen embryos (including yolk sac), fetal livers, and adult blood (animals >8 weeks old) and analyzed by S1 nuclease protection assays as described previously (Fraser et al. 1990). Quantitation of signals was performed using a PhosphorImager (Molecular Dynamics). S1 probes (Lindenbaum and Grosveld 1990; Milot et al. 1996) were end-labeled with T4 polynucleotide kinase and equimolar amounts of each end-labeled probe were used per reaction. Amounts of RNA analyzed per protection assay: day 10 and 11 embryos, 6 μg; day 11 fetal livers, 3 μg; day 12 fetal livers, 2 μg; day 13, 14, 15, and 16 fetal livers, 1 μg; adult blood, 1 μg.

Primary transcript in situ hybridization

Embryonic blood (10.5-day) and 12.5- and 13.5-day fetal livers were disrupted in PBS. Cells were spotted and immobilized onto poly-L-lysine-coated slides and subsequently fixed in 4% formaldehyde, 5% acetic acid in saline for 20 min at room temperature. Slides were further processed and used for in situ hybridization analysis and antibody detection as described previously (Wijgerde et al. 1995). Probes were labeled with digoxygenin, biotin, or dinitrophenol. Transcription signals were quantitated by counting cells (see Table 1) using an epifluorescence microscope.

Isolation of nuclei and DNase I hypersensitive site analysis

Nuclei were isolated from 13.5-day frozen fetal livers as described by Forrester et al. (1990). For each genotype ($EKLF^{+/+}$, $EKLF^{-/-}$) 10 livers were disrupted with 10 strokes of a type B pestle. Aliquots (100-μl) were digested with increasing amounts of DNase I for 3 min at 37°C. Two aliqouts, one incubated on ice and one at 37°C, were devoid of any DNase I and served as controls for the zero time point and endogenous nucleases, respectively. Reactions were treated with 250 μg/ml proteinase K in 0.3 M NaCl, 0.5% SDS, 5 mM EDTA, and 10 mM Tris at pH 8 at 55°C for 16 hr, phenol/chloroform extracted and ethanol precipitated. Pellets were dissolved in 80 μl of distilled water. Twenty microliters of DNA was digested with appropriate restriction enzyme and analyzed via Southern blot. The following restriction enzymes and probes were used: γ promoter, *Eco*RI digest, *Bam*HI–*Eco*RI γIVSII fragment; β promoter, *Eco*RI digest, *Bam*HI–*Eco*RI βIVSII fragment; human HS1, *Pst*I, 520-bp *Hin*cII fragment; human HS2, *Pst*I, 1.5-kb *Kpn*I–*Bgl*II; human HS3, *Hin*dIII, 1.9-kb *Hin*dIII; human HS4, *Hin*dIII digest, 600-bp *Bam*HI–*Sac*I; β-maj promoter, *Eco*RI, 1-kb *Eco*RI–*Hin*dIII; mouse HS3, *Hin*dIII digest, 1.9-kb *Hin*dIII; mouse α1 and α2, *Sac*I digest, 600-bp *Bam*HI–*Sac*I. The relative hypersensitivity between knockout and wild-type lines was quantitated via PhosphorImage analysis. The ratio of hypersensitive bands over parental band was plotted and the slopes of the resulting lines were compared.

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