The active spatial organization of the \(\beta\)-globin locus requires the transcription factor EKLF

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Three-dimensional organization of a gene locus is important for its regulation, as recently demonstrated for the \(\beta\)-globin locus. When actively expressed, the cis-regulatory elements of the \(\beta\)-globin locus are in proximity in the nuclear space, forming a compartment termed the Active Chromatin Hub (ACH). However, it is unknown which proteins are involved in ACH formation. Here, we show that EKLF, an erythroid transcription factor required for adult \(\beta\)-globin gene transcription, is also required for ACH formation. We conclude that transcription factors can play an essential role in the three-dimensional organization of gene loci.

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The mouse \(\beta\)-globin locus contains multiple \(\beta\)-like globin genes, arranged from 5' to 3' in order of their developmental expression (Fig. 1A). The adult-type \(\beta\max\)-gene is transcribed at a very low level during primitive erythropoiesis in the embryonic yolk sac, but becomes expressed at high levels around day 11 of gestation (E11) when definitive erythropoiesis commences in the fetal liver (Trimborn et al. 1999). The \(\beta\)-globin locus control region (LCR) is essential for efficient globin transcription (Grosveld et al. 1987; Bender et al. 2000). It consists of a series of DNaseI hypersensitive sites (HS) located ∼50 kb upstream of the \(\beta\max\) promoter (Fig. 1A). We have shown that the \(\beta\)-globin locus forms an Active Chromatin Hub (ACH) in erythroid cells (Tolhuis et al. 2002). The ACH is a nuclear compartment dedicated to RNA polymerase II transcription, formed by the cis-regulatory elements of the \(\beta\)-globin locus with the intervening DNA looping out. The ACH consists of the HS of the LCR, two HS located ∼60 kb upstream of the embryonic \(e\)-globin gene \((5^{HS}\sim62,60)\) and 3'HS1 downstream of the genes. In addition, the actively expressed globin genes are part of the ACH (Carter et al. 2002; Tolhuis et al. 2002). In erythroid precursors that do not express the globin genes yet, a substructure of the ACH, called a chromatin hub (CH) [Patrinos et al. 2004] is found, which excludes the genes and the HS at the 3' site of the LCR (Palstra et al. 2003).

Expression of the \(\beta\max\)-gene requires the presence of the erythroid Krüppel-like transcription factor EKLF, the erythroid-specific member of the Sp/XKLF-family [Miller and Bieker 1993]. EKLF\(^{-/-}\) mice die of anemia around E14, because of a deficit in \(\beta\)-globin expression (Nuez et al. 1995, Perkins et al. 1995). The \(\beta\)-globin locus contains a number of EKLF-binding sites, in particular in the LCR and the \(\beta\max\)-globin promoter [Perkins 1999, Bieker 2001]. Because \(\beta\max\)-globin expression depends on the presence of EKLF, we were interested in determining whether EKLF is involved in the formation of the ACH.

Results and Discussion

We used chromatin conformation capture (3C) technology (Dekker et al. 2002) to investigate the three-dimensional conformation of the mouse \(\beta\)-globin locus in the absence of EKLF. Cell nuclei from E12.5 fetal liver were cross-linked with formaldehyde, followed by restriction enzyme digestion of the DNA. The samples were ligated under conditions that favor the ligation of DNA fragments that are physically connected through the cross-links. Quantitative PCR across the junctions is used to determine the relative cross-linking frequencies between restriction fragments in the locus. This provides an indication of the nuclear proximity of DNA fragments in vivo [Dekker et al. 2002, Tolhuis et al. 2003]. Cross-linking frequencies were determined for a total of 66 junctions that can be formed between 12 selected HindIII fragments spread over ∼170 kb of DNA encompassing the \(\beta\)-globin gene cluster (Fig. 1). Examples of quantitative PCR reactions with some of the primer combinations are shown in Figure 1B. An overview of the locus-wide cross-linking frequencies of a restriction fragment that contains the \(\beta\max\) promoter is shown in Figure 1C. The brain serves as a nonexpressing control tissue (black curve), in which the \(\beta\)-globin locus appears to adopt a linear conformation [Tolhuis et al. 2002]. In wild-type E12.5 fetal liver cells, high cross-linking frequencies are found with the LCR and \(5^{HS}\sim62\), indicating their proximity to the \(\beta\max\) promoter in vivo (blue curve). In the absence of EKLF however, these cross-linking frequencies are much lower and no interaction with a distal site stands out clearly (red curve), showing that the \(\beta\max\) promoter does not participate stably in a spatial clustering of chromatin. A comparable pattern is observed with locus-wide cross-linking frequencies of a fragment containing \(5^{HS}\sim62\) (Fig. 1D). Together with \(5^{HS}\sim62\), the most prominent transcriptional activating element of the LCR (Ellis et al. 1993, 1996; Fraser et al. 1993; Fiering et al. 1995, Hug et al. 1996), interactions with 3'HS1, and the other HS of the LCR are strongly reduced in the absence of EKLF, indicating that \(5^{HS}\sim62\) requires the presence of EKLF to participate in the ACH.

The results shown in Figure 1 demonstrate that the complete ACH is not formed in the absence of EKLF. However, the observed cross-linking frequencies in EKLF\(^{-/-}\) fetal liver cells are still higher than those found in nonexpressing brain cells, indicating a different, non-linear, structure. To investigate this, we compared the locus-wide cross-linking frequencies of restriction frag-
Results obtained with wild-type livers are shown in blue, indicated by green rectangles and are numbered. DNaseI HS are shown as red ovals with arrows. The scale is in kilobases. (PCR-amplified ligation products run on a 2% agarose gel. Primer combinations are shown on the right.) Examples of PCR-amplified ligation products run on a 2% agarose gel. Primer combinations are shown on the right. XPB is used to standardize the amount of template (Palstra et al. 2003). (+/+ Wild type; −/− EKLF knockout. (C,D) Locus-wide relative cross-linking frequencies in E12.5 fetal livers. Results obtained with wild-type livers are shown in blue, EKLF−/− livers in red, nonexpressing brains in black. The X axis shows position in the locus. Gray shading indicates the positions and sizes of the HindIII fragments containing primers used in the PCR analysis. Black shading represents the position of the fragment containing the fixed primer in the HindIII fragment of the βm5-gene (C) or 5′HS2 (D). Within each graph, the highest cross-linking frequency value is set to 1. Error bars indicate S.E.M.

There are remarkable similarities between the structure of the β-globin locus in EKLF−/− fetal liver cells and that observed in I/11 erythroid progenitor cells that do not yet express globin (Palstra et al. 2003; Supplementary Fig. 1). This suggests that EKLF is required for progression from the chromatin hub present in erythroid precursors to a fully active ACH. To investigate whether this β-globin structure in EKLF null cells is a direct consequence of EKLF deficiency or caused by a general differentiation failure, we analyzed expression of the erythroid-specific, EKLF-independent, α-globin locus. The mouse α-globin locus has two active genes in the fetal liver, α1 and α2, and contains a HS 26 kb upstream of the β-globin promoter that is similar to the human α-globin enhancer HS-40 (Fig. 3B; Flint et al. 2001). It is likely that, analogous to the LCR, this element will interact with the α-like globin promoters to enhance expression. The cross-linking frequencies of the restriction fragments containing HS-26 and α2-globin are shown in Figure 3C and D. In wild-type and EKLF−/− fetal liver cells, the cross-linking frequencies are clearly higher than those observed in nonexpressing brain tissue, indicating that HS-26 and the α2-globin gene are in close proximity in both types of erythroid cells. The slightly reduced interaction frequencies observed in EKLF knockout compared with wild-type fetal liver can be explained by the 20% reduction of α-globin expressing cells [see above]. We conclude that major alterations in spatial organization are restricted to the EKLF-dependent β-globin locus.

To further investigate whether changes in the spatial organization of the β-globin locus are a direct effect of...
the activity of EKLF, we wished to induce EKLF activation and simultaneously prevent it from activating secondary pathways. For this, we used a fusion between EKLF and a modified estrogen-receptor ligand-binding domain [EKLF–lbd protein], which can be activated by 4-hydroxy-tamoxifen (4-OHT) (Littlewood et al. 1995). We wanted to test whether, in an EKLF null background, activated EKLF–lbd protein restores ACH formation in the presence of the protein synthesis inhibitor cycloheximide (CHX). In such a setup, genes activated by EKLF cannot be translated into protein, and therefore any structural changes would have to be attributed to EKLF acting directly on the β-globin locus. Transgenic mice carrying an expression construct of an EKLF–lbd fusion protein were generated. To ensure expression of the fusion protein in EKLF null erythroid cells, we used the erythroid-specific pEV3 expression vector [Needham et al. 1992] and replaced the β-globin promoter with the α-globin promoter. Western blot analysis demonstrates the presence of the HA-tagged EKLF–lbd fusion protein [Fig. 4A]. We have previously shown that an EKLF–pEV3 transgene rescues the EKLF null mutation [Tewari et al. 1998]. To test whether uninduced EKLF–lbd fusion protein is inactive, we crossed the EKLF–lbd transgenics with the EKLF knockout mouse. No EKLF null::EKLF–lbd transgene pups were born. When we dissected the fetuses resulting from this cross at E12.5, we found that the EKLF null::EKLF–lbd transgenic fetuses were indistinguishable from EKLF null fetuses, for example, displaying signs of severe anemia and having very pale fetal livers [data not shown]. We conclude that the EKLF–lbd fusion protein is inactive and does not rescue the EKLF null mutation.

To test the ability of activated EKLF–lbd fusion protein to rescue β-globin gene transcription, we cultured EKLF null::EKLF–lbd fetal liver cells in the presence of 4-OHT [Fig. 4B]. After 16 h of culturing, a subset of the cells was used to check for the activation of β-globin gene expression. Real-time RT–PCR analysis of steady-state mRNA levels shows that the β-globin gene is activated in EKLF null::EKLF–lbd cells in the presence of 4-OHT (Fig. 4C). The amount of β-globin transcripts in the tamoxifen-rescued cells is much lower than in wild-type cells, which is not surprising, as the former cells just start to accumulate β-globin mRNA levels. We conclude that the EKLF–lbd fusion protein can be induced with 4-OHT to activate β-globin gene expression. Moreover, β-globin gene activation by 4-OHT-induced EKLF–lbd also occurs in the presence of CHX (Fig. 4C).

The remaining cells were subjected to 3C analysis using a procedure modified for use with small numbers of cells. Because the amount of material was limiting, we focused on the analysis of interactions between 5′HS2, one of the most prominent activating elements of the
Recent work has shown that deletion of the promoter of the adult β-globin gene in the human β-globin locus mildly affects ACH formation, suggesting that in addition to the β-globin promoter, other cis-regulatory elements in the human β-globin locus are involved in these interactions [Patrinos et al. 2004]. EKLF-binding sites are also present in the LCR, in particular in 5′HS3, and in the 3′ enhancer of the β-globin gene (Wall et al. 1988; Gillemans et al. 1998). Together, these data suggest that the EKLF-dependent interactions of the adult β-globin genes with the ACH involve multiple cis-regulatory elements.

It is also interesting to note that in the EKLF knockout, absence of spatial interactions coincides with loss of chromatin accessibility at 5′HS3 and the βmax-promoter [Wijgerde et al. 1996; De Laat and Grosveld 2003]. We conclude that EKLF is necessary for hypersensitive site formation and the participation of the LCR and the β-globin promoter in the ACH, probably through interactions with a SWI/SNF-related chromatin remodeling complex [Armstrong et al. 1998]. Thus, EKLF is the first example of a transcription factor that is required for the proper spatial organization of a mammalian gene locus.

Materials and methods

**Chromosome conformation capture**

EKLF<sup>−/−</sup> mice (Nuez et al. 1995) were crossed, and E12.5 fetal livers and brains were isolated. 3C analysis was performed as described [Splinter et al. 2004], with minor adjustments. Individual liver and brain samples were subjected to formaldehyde cross-linking. HindIII restriction enzyme digestion of cross-linked DNA, intramolecular ligation, reversal of cross-links, PCR analysis of ligation products, and calculation of relative cross-linking frequencies was done with 15 pooled wild-type fetal livers, 15 EKLF<sup>−/−</sup> fetal livers and cells of three pooled EKLF<sup>−/−</sup> brains. Two independent samples were prepared for the analysis. Each PCR reaction was performed in duplicate and repeated at least three times.

**α-Globin**

HS-26-a2 promoter cross-linking frequencies were determined with the DNA samples described above and primers recognizing the HindIII restriction fragment containing HS-26 (5′-GAACTTCCATCTCTCAAGGG-3′) and the α2 promoter (5′-AAGAGGTCAGTTATCTACTG-3′). In situ hybridization of E12.5 fetal liver cells was performed as described before [Van de Corput and Grosveld 2001]. Cells were scored positive if α-globin mRNA, primary transcript, or both, was detected. Greater than 300 cells were counted to determine the percentage of α-globin-positive cells in each sample.

**Generation of EKLF-<i>lbd</i> transgenic mice**

A DNA fragment containing EKLF cDNA and the first intron was linked in frame with the HA tag sequence at the 5′ and the lbd-coding sequence at the 3′ side. This construct was cloned into the pE3 vector [Neddelham et al. 1992] and the β-promoter was replaced by a fragment containing the α-globin promoter. The vector was linearized by Aattl, and transgenic mice were generated as described [Kollins et al. 1986].

**Culture of primary fetal liver cells**

Livers were isolated from E12.5 control and EKLF<sup>−/−</sup> fetal livers. The genotype of the fetuses was confirmed by PCR. Single-cell suspensions of individual fetal livers were cultured for 16h in StemPro-34 containing 1% BSA, 1% glutamine, and 10 U/mL epo, but without serum supplement. The EKLF-lbd was activated by supplementing the medium with either 250 nM 4-hydroxy-tamoxifen (4-OHT) alone or with 250 nM 4-hydroxy-tamoxifen (4-OHT) alone or with 250 nM
Figure 5. The formation of the complete ACH requires the presence of EKLF. A two-dimensional representation of the proposed three-dimensional structure of the ACH is shown. The ACH is a nuclear compartment dedicated to RNA polymerase II transcription, formed by cis-regulatory elements of the β-globin locus (Palstra et al. 2003). In erythroid cells, a substructure of the ACH, consisting of 5′HS-62/-60, 3′HS1 and HS at the 5′ side of the LCR, is formed independently of EKLF. Progression of this substructure to a fully functional ACH, including the HS at the 3′ side of the LCR and the active β-globin gene, is dependent on the presence of EKLF. (Gray sphere) ACH substructure; (yellow sphere) ACH. RNA transcripts are indicated as red lines. See the legend for Figure 1A for other details.

Preparation of cDNA and Real-time PCR
RNA was isolated using Trizol, according to the manufacturer’s guidelines [Invitrogen]. The Super-script reverse transcriptase Kit [Invitrogen] was used for preparation of oligo-dT primed cDNA. Expression levels were determined on the Bio-Rad I-Cycler using the qPCR Core kit for Sybr Green 1 [Eurogentec]. Expression levels of Hprt were used for normalization of β-globin expression levels.

Primers used were as follows: Hprt-s, AGGCCCTAAGGATGCCAGGCAAGT; Hprt-as, ATGGCCCATAGGACTAGAACA; β-major-s, ATGCCAAAAGTGGAGCCCAT; β-major-as, CCCAGCAATCCAGGAT.

Preparation of 3C templates
For the limiting number of cells (∼1.10^6) obtained from the individual EKLF null/−EKLF−/−tg fetal livers, we adapted the previously described protocol [Tolluis et al. 2002].

Cross-linked nuclei of E12.5 fetal livers were resuspended in 50 µL of digestion buffer containing 0.1% SDS and incubated for 1 h at 37°C with agitation. Triton X-100 was added to 1.2%, and the nuclei were further incubated for 1 h at 37°C.

The cross-linked chromatin was digested overnight at 37°C with 10 U of HindIII. The restriction enzyme was heat inactivated (25 min at 65°C). After addition of 200 µL of 1.25x ligase buffer and 40 U of T4 ligase, the chromatin was ligated for 4.5 h at 16°C, followed by 30 min at room temperature. Proteinase K was added, and samples were incubated overnight at 65°C to reverse the cross-links. The following day, samples were incubated for 30 min with RNaseA, and the DNA was purified by phenol extraction and ethanol precipitation using glycogen as a carrier. Locus-wide cross-linking frequencies of wild-type fetal livers treated with this adapted protocol were similar to those found previously [data not shown].

PCR analysis of the ligation products was performed as described before [Tolluis et al. 2002; Palstra et al. 2003].

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EKF and 3D organization of globin locus

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