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# The β-globin nuclear compartment in development and erythroid differentiation

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**Efficient transcription of genes requires a high local concentration of the relevant *trans*-acting factors. Nuclear compartmentalization can provide an effective means to locally increase the concentration of rapidly moving *trans*-acting factors; this may be achieved by spatial clustering of chromatin-associated binding sites for such factors[1–5]. Here we analyze the structure of an erythroid-specific spatial cluster of *cis*-regulatory elements and active β-globin genes, the active chromatin hub (ACH; ref. 6), at different stages of development and in erythroid progenitors. We show, in mice and humans, that a core ACH is developmentally conserved and consists of the hypersensitive sites (HS1–HS6) of the locus control region (LCR), the upstream 5′ HS–60/–62 and downstream 3′ HS1. Globin genes switch their interaction with this cluster during development, correlating with the switch in their transcriptional activity[7]. In mouse erythroid progenitors that are committed to but do not yet express β-globin, only the interactions between 5′ HS–60/–62, 3′ HS1 and hypersensitive sites at the 5′ side of the LCR are stably present. After induction of differentiation, these sites cluster with the rest of the LCR and the gene that is activated. We conclude that during erythroid differentiation, *cis*-regulatory DNA elements create a developmentally conserved nuclear compartment dedicated to RNA polymerase II transcription of β-globin genes.**

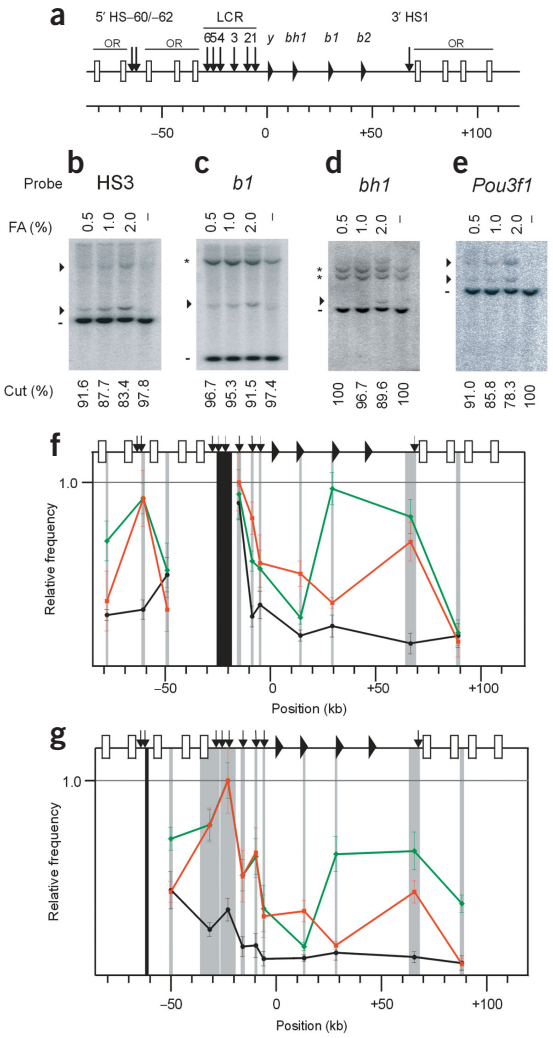


**Figure 1** Spatial organization of the mouse β-globin locus. (**a**) Schematic presentation of the mouse locus. Arrows indicate the individual hypersensitive sites, triangles indicate globin genes and boxes indicate the olfactory receptor (OR) genes. (**b–e**) Southern blots show that in definitive erythrocytes, digestion efficiency of crosslinked chromatin depends on formaldehyde concentration and is comparable between a hypersensitive site in the LCR (**b**), a transcribed gene in the locus (**c**), a nonexpressed gene in the locus (**d**) and a nonexpressed gene on a different chromosome (Chr. 4; **e**). Percentage formaldehyde crosslinking (FA %) is shown at the top of each blot (– indicates genomic DNA not treated with formaldehyde), and the yield of specifically cut fragments is shown at the bottom (in percentages). Arrowheads indicate partial digests, and asterisks indicate crosshybridization signals with other genes. (**f**,**g**) Erythroid-specific and developmentally stable clustering of *cis*-regulatory elements. Relative crosslinking frequencies observed in primitive erythrocytes are shown in red, definitive erythrocytes in green and nonexpressing brain in black. Gray shading indicates position and size of the analyzed fragments, and black shading represents the 'fixed' fragments HS4–HS5 (**f**) and 5′ HS –60/–62 (**g**). In each graph, the highest crosslinking frequency value was set to 1. The *x* axis shows position in the locus. Errors bars represent s.e.m.

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The mouse and human β-globin loci contain an upstream LCR and multiple β-like genes arranged from 5′ to 3′ in order of their developmental expression (**Fig. 1a**). In addition there are several distal hypersensitive sites, including a downstream 3′ HS1 (approximately 20-kb 3′ of the β-globin genes) and two upstream hypersensitive sites, ~60 kb (mouse) and ~110 kb (human) away from the genes[8]. The loci are embedded in an olfactory receptor gene cluster that is inactive in erythroid cells[9]. To investigate the spatial organization of β-globin loci in mice and humans during development and erythroid differentiation, we applied chromosome conformation capture (3C) technology[6,10]. 3C technology involves quantitative PCR analysis of crosslinking frequencies between two given DNA restriction fragments, which gives a measure of their proximity in the nuclear space. Local chromatin configuration has no effect on digestion efficiency, implying that the assay is not biased owing to preferential restriction enzyme digestion of one site over the other (**Fig. 1b–e**; for other controls, see Methods and ref. 6).

First, we analyzed the spatial organization of the mouse β-globin locus in primitive erythroid cells present in blood from embryos at 10.5 d post-coitum (d.p.c.), which predominantly express the embryonic globin genes *Hbb-y* and *Hbb-bh1* (ref. 11). We determined crosslinking frequencies for 66 pairs of *Hin*dIII restriction fragments, spread over ~170 kb of DNA encompassing the mouse β-globin gene cluster. The 3C measurements indicate a basic structural organization in primitive cells very similar to that observed previously in definitive blood cells isolated from 14.5-d.p.c. fetal liver[6]. This is best illustrated by comparing the locus-wide crosslinking frequencies of a restriction fragment that contains HS4–HS5 of the LCR. Two peaks of high crosslinking frequency with this genomic site stand out in primitive blood cells: one with the upstream HS–60/–62 and another with 3′ HS1 downstream of the genes (**Fig. 1f**). We found significantly lower crosslinking frequencies with fragments in between, suggesting that the LCR interacts with these distal hypersensitive sites through looping. We observed the same interactions in definitive blood cells that exclusively express the adult globin genes *Hbb-b1* and *Hbb-b2* (ref. 11), where *Hbb-b1* is also found in close proximity (**Fig. 1f**).

In contrast, in nonexpressing brain cells, HS4–HS5 has no peaks of interaction with distal DNA fragments, suggesting a linear conformation of the transcriptionally inactive locus[6]. We obtained similar results when analyzing the locus-wide crosslinking frequencies of fragments carrying 5′ HS–60/–62 (**Fig. 1g**) and other hypersensitive sites (data not shown): interactions among the *cis*-regulatory elements of the β-globin locus were conserved between primitive and definitive erythroid cells. We conclude that the *cis*-regulatory elements of the mouse β-globin locus spatially cluster to form a transcription regulatory compartment that is conserved between primitive and definitive erythroid cells, two developmentally different types of cells that express a different subset of β-like globin genes. This core ACH includes the two hypersensitive sites at –60 kb, all hypersensitive sites of the LCR and 3′ HS1.

The main differences in conformation between the two expressing cell types seem to be confined to interactions between the globin genes and the regulatory DNA elements. We confirmed this by measuring crosslinking frequencies with HS2 and HS3 of the LCR, the two most prominent transcriptional activating elements[12–17]. The embryonic globin genes *Hbb-y* and *Hbb-bh1* interacted frequently with these elements in primitive erythroid cells, whereas in definitive red blood cells interaction frequencies between these sites dropped to levels similar to those observed in the inactive brain (**Fig. 2**). We found the opposite pattern for the adult genes *Hbb-b1* and *Hbb-b2*, which interacted most

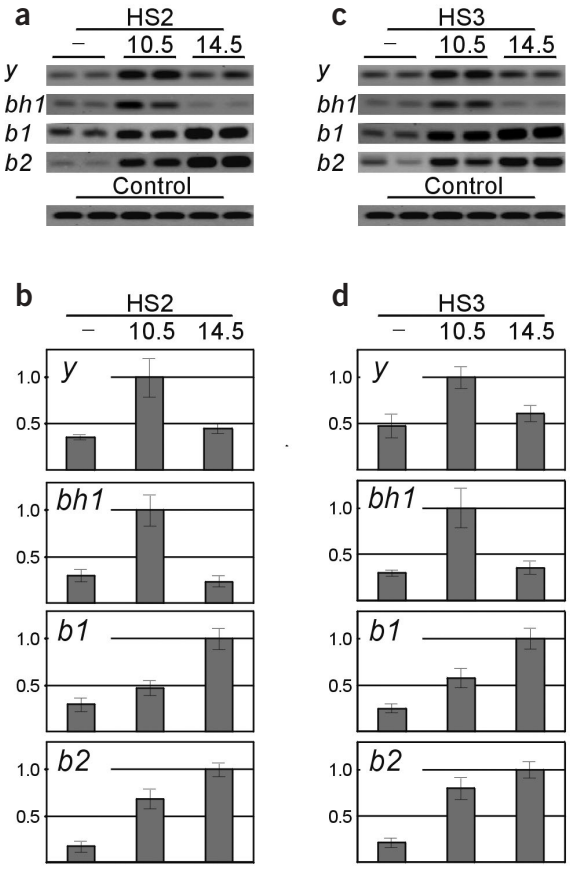


**Figure 2** A developmental switch occurs in contacts between individual β-globin genes and the core ACH of the mouse β-globin locus. (**a**,**b**) Crosslinking frequencies of HS2 and the β-globin genes were measured. An example of PCR-amplified ligation products on 2% agarose gel (**a**) and the quantified data of all experiments (at least five in duplicate per primer set; **b**) are shown. (**c**,**d**) Similar to **a** and **b** but for HS3 and the β-globin genes. Error bars in **b** and **d** represent s.e.m. –, nonexpressing brain; 10.5, primitive erythrocytes; 14.5, definitive erythrocytes. Control is PCR-amplified ligation product of two restriction fragments in the *Ercc3* locus. Crosslinking frequencies shown in **b** and **d** are not corrected for PCR amplification efficiency; therefore, only signals obtained with the same primer set can be compared.

frequently with HS2 and HS3 in definitive erythroid cells. Crosslinking frequencies between these sites in 10.5-d.p.c. embryonic blood were not as low as in brain, probably owing to the fact that *Hbb-b1* and *Hbb-b2* are already transcriptionally active at this stage, albeit at less than 10% of the levels observed in definitive cells[11]. Alternatively, it may merely be the result of 3′ HS1 interacting with the LCR and the adult genes being dragged along, as we previously found that the region between *Hbb-b2* and 3′ HS1, which is full of repetitive sequences, acts as a rigid region[6]. These data show that there is a developmental switch in contacts between the different globin genes and a core ACH created by regulatory elements that surround the genes in *cis*. This structural change correlates with the developmental switch in expression of the genes.

We next analyzed the conformation of the human β-globin locus at different stages of development. The mouse and human β-globin gene loci have a high degree of nucleotide sequence conservation, particularly at regions implicated in gene regulation[9,18]. We used transgenic mice carrying a single copy of a 185-kb PAC (**Fig. 3a**)

spanning the human β-globin locus that had a normal expression pattern (refs. 19,20 and G. Patrinos and F.G., unpublished data). Though large, this PAC does not include the human equivalent of the mouse 5′ HS–60/–62, which is located ~110 kb upstream of the human globin genes[8,9]. We analyzed the conformation of the transgenic human globin locus in 10.5-d.p.c. embryonic blood, 14.5-d.p.c. fetal liver and 14.5-d.p.c. fetal brain, measuring almost all of the 120 site pairs that can be formed between the 16 *Eco*RI fragments that we analyzed. The locus-wide crosslinking frequencies of a fragment corresponding to 3′ HS1 illustrate that the transgenic human locus also forms a core ACH, consisting of the 3′ HS1 and the hypersensitive site of the LCR, that is conserved in primitive and definitive erythroid cells (**Fig. 3b**). The structural changes we observed primarily concerned the position of the genes relative to this core ACH, correlating with transcriptional activity. Thus, the embryonic gene *HBE1* and the two *HBG* genes most frequently interact with HS2–HS4 (**Fig. 3c–e**) and 3′ HS1 (**Fig. 3b**) in primitive erythroid cells, and the adult gene *HBB* primarily contacts the ACH in definitive cells (**Fig. 3c–e**). We found identical results for a *Hin*dIII digest and for a different transgenic PAC line (data not shown). Results obtained with definitive erythroid cells isolated from adult bone marrow (Ter119$^+$) were identical to those found for 14.5-d.p.c. fetal liver cells (data not shown). The lower crosslinking frequency of HS5 in the definitive cells is notable, as we have recently shown that this element has LCR-blocking activity in primitive but not definitive erythroid cells[21]. We conclude that the overall spatial organization of the β-globin gene cluster is conserved from mice to humans.

Next we determined β-globin genomic site interactions in I/11 erythroid progenitor cells that are committed to but do not yet express the β-globin genes. When exposed to physiologically relevant stimuli, I/11 cells synchronously undergo the normal *in vivo* differentiation program to mature terminally into enucleated erythrocytes[22,23]. As expected, in differentiating I/11 cells that actively transcribe the adult β-like globin genes, the locus adopted a spatial organization very similar to that observed previously in definitive erythroid cells isolated from fetal livers[6] (**Fig. 4**). In uninduced proliferating I/11 cells that do not yet express the β-globin genes, however, we observed a different structure. Locus-wide crosslinking frequencies of a fragment corresponding to the gene *Hbb-b1* were lower than those observed in erythroid cells expressing the gene (**Fig. 4a**). We observed a similar reduction in locus-wide crosslinking frequencies for a fragment containing HS2 and for most other fragments (data not shown).

The structure of the locus poised for transcription was clearly different from that of the inactive locus in brain cells. This structure is better resolved by looking at the locus-wide crosslinking frequencies of the restriction fragment that contains HS4–HS5 of the LCR. Two peaks of high crosslinking frequency with this fragment stand out in erythroid progenitor cells: one with 5′ HS–60/–62 and another with 3′ HS1 (**Fig. 4b**). Interactions among these three sites occur almost as frequently in proliferating progenitors as in differentiating erythroid cells, whereas all other interactions examined between globin site pairs are much less frequent in progenitor cells (**Fig. 4** and data not shown). We conclude that the β-globin locus that is poised for transcription in progenitor cells adopts a looped conformation through frequent interactions between the two distal regulatory elements at either end of the locus (5′ HS–60/–62 and 3′ HS1) and hypersensitive sites at the 5′ side of the LCR (HS4, HS5 or HS6; it is not clear which of these hypersensitive sites is responsible for direct interaction). After induction of differentiation, clustering with the active genes and the complete LCR is established and the β-globin genes are expressed (**Fig. 5**).

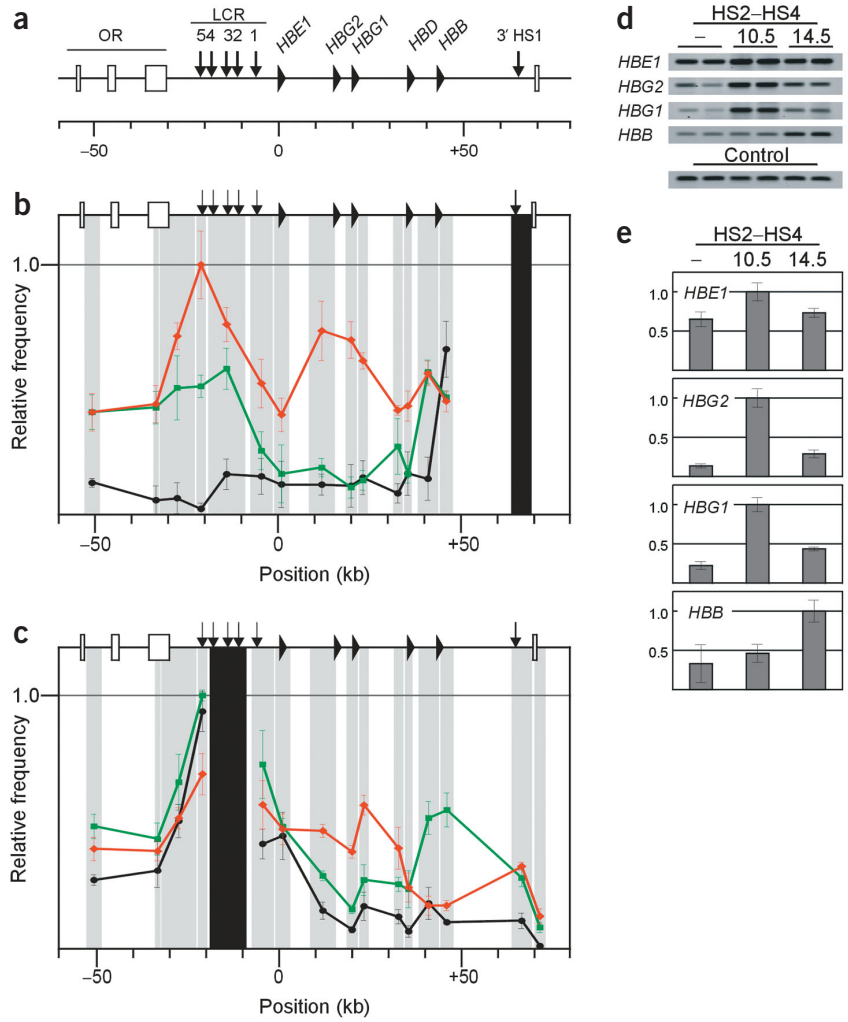


**Figure 3** Spatial organization of the human β-globin locus. (**a**) Schematic presentation of the human locus. (**b**) Locus-wide crosslinking frequencies of a 3′ HS1 fragment show erythroid cell–specific clustering with the LCR throughout development. (**c**) Developmental switching in contacts of the LCR between the different β-globin genes as shown by locus-wide crosslinking frequencies of HS2–HS4. (**d**,**e**) The contacts between HS2–HS4 of the LCR and individual β-globin genes alter during development in erythroid cells, as shown by an example on agarose gel (**d**) and quantified data (**e**; at least five experiments in duplicate per primer set). Controls, symbols, color patterns and numbering are as in **Figures 1** and **2**.

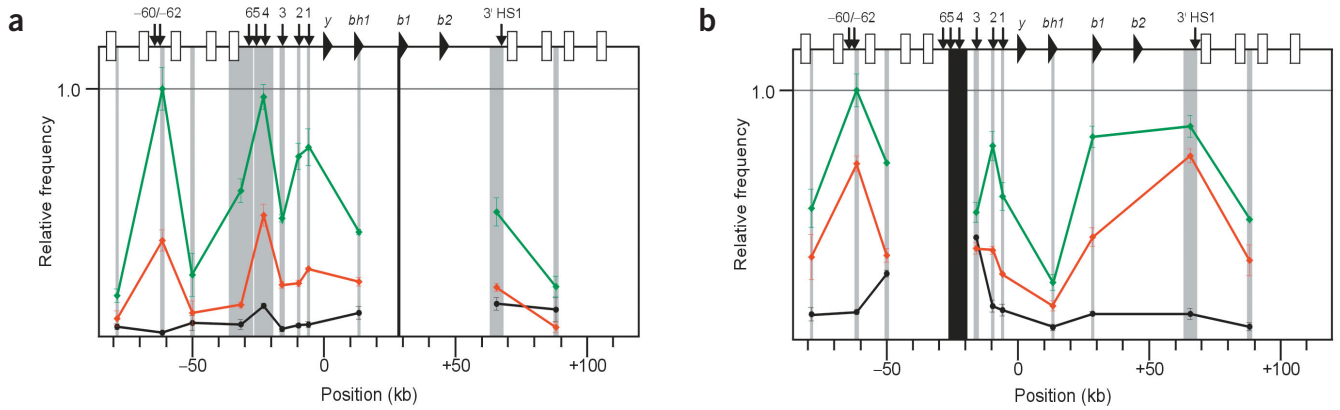


**Figure 4** Spatial organization of the mouse β-globin locus in erythroid progenitors. Controls, symbols and numbering are as in **Figure 1**, black lines represent brain, red lines represent proliferating I/11 erythroid progenitor cells and green lines represent differentiated I/11 cells. (**a**) Locus-wide crosslinking frequencies of *Hbb-b1*. (**b**) Locus-wide crosslinking frequencies of HS4–HS5. Only the interactions among HS4–HS5, 5′ HS–60/–62 and 3′ HS1 are already fully established in nonexpressing progenitor cells.

In summary, our data strongly suggest that regulatory elements surrounding the β-globin genes in *cis* create an erythroid-specific developmentally stable nuclear compartment dedicated to RNA polymerase II transcription (**Fig. 5**). A substructure is already present in erythroid progenitors that do not express globin, and notably, the three sites involved in this structure all bind CTCF[24]. The 3′ HS1 and 5′ HS–60/–62 are dispensable for normal globin gene expression in transgenic mice[19,21], suggesting that these sites have a more general structural role not related to transcription per se.

Spatial clustering of transcription regulatory elements results in a high local concentration of DNA binding sites for cognate transcription factors, which consequently accumulate at the site. Efficiency of transcription is proportional to the concentration of transcription factors involved, and in agreement, we found that proximity of β-globin genes to the ACH correlated with transcriptional activity. The paradigm of a chromatin-associated nuclear compartment is the nucleolus, dedicated to RNA polymerase I transcription of ribosomal RNA genes[3]. No polymerase II–dependent gene-specific compartments have been described before, but a precedent for this was provided by electron microscopy studies showing that RNA polymerase II clusters in discrete transcription factories in the nucleus[25,26]. The fact that the density of RNA polymerases on active β-globin and ribosomal RNA genes is much higher than on most other active genes[27,28] suggests that such nuclear compartments formed by numerous chromatin-associated regulatory elements primarily function to increase the efficiency of transcription.

## METHODS

**Chromosome conformation capture (3C).** We carried out isolation and formaldehyde fixation of primary cells, restriction enzyme digestion of crosslinked DNA in the nucleus, intramolecular ligation, reversal of crosslinks, PCR analysis of ligation products and calculation of relative crosslinking frequencies as described before[6,10], with some modifications. Before fixation, we forced cells obtained from embryonic blood (from 10.5-d.p.c. embryos) and from fetal liver and fetal brain (both from 14.5-d.p.c. embryos; $4 \times 10^7$ cells per tissue) through a cell-strainer cap (Falcon #352235) to obtain a homogeneous single-cell suspension. To correct for differences in quality and quantity of template, we normalized ligation frequencies between globin site pairs to those detected between two restriction fragments (with the sites analyzed 8.3 kb apart) in the *Ercc3* locus (instead of the previously used *Calr* locus[6]). *Ercc3* encodes a subunit of the basal tran-

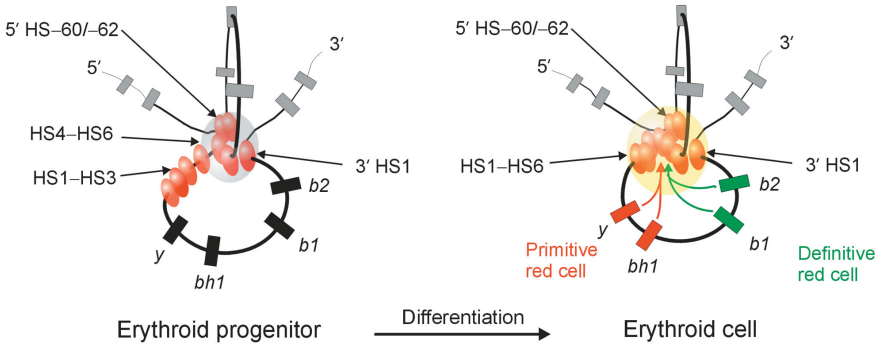


**Figure 5** *Cis*-regulatory elements of the β-globin locus create a nuclear compartment dedicated to RNA polymerase II transcription: the active chromatin hub. Two-dimensional presentation of three-dimensional interactions that occur between regulatory DNA elements 130 kb apart (red ovals) and β-globin genes (active, red and green rectangles; inactive, black rectangles) in erythroid progenitors (left) and in differentiated primitive and definitive erythroid cells (right). In erythroid progenitors not expressing globin, a substructure (gray sphere) is present that is formed through interactions between the upstream 5′ HS–60/–62, the downstream 3′ HS1 and hypersensitive sites at the 5′ side of the LCR (HS4–HS6; it is not yet clear which of these hypersensitive sites is directly responsible for this interaction). During erythroid differentiation, the β-globin gene that gets activated and the rest of the LCR stably interact with this substructure to form a functional ACH (yellow sphere); β-globin gene expression is activated. Clustering of binding sites for transcription factors in the ACH causes local accumulation of cognate proteins and associated positive chromatin modifiers, required to drive efficient transcription of the globin genes. The core of the ACH is erythroid-specific and developmentally stable; a developmental switch occurs in globin genes entering this nuclear compartment, as indicated by the arrows. Inactive globin and olfactory receptor genes (gray squares) loop out.

scription factor TFIIH, and we assumed that expression levels and spatial conformation of this gene were similar in all analyzed tissues. To compare signal intensities obtained with different primer sets in a quantitative manner, we included a control template containing all possible ligation products in equimolar amounts to correct for the PCR amplification efficiency of each set. For this purpose we used BAC and PAC clones spanning the complete loci (instead of the previously used PCR fragments that span the restriction sites of interest[6]). For the mouse β-globin locus we used a 214-kb BAC (#RP23-370E12, Ensembl Genome Browser), and for the human β-globin locus we used a 185-kb PAC[20]. In addition, we used a 60- to 70-kb PAC containing the mouse *Ercc3* locus (PAC Clone #443-C18, MRC geneservice). We mixed either the mouse globin BAC or the human globin PAC with the *Ercc3* PAC at equimolar amounts. We then digested and ligated the mixes as described[6]. We could not obtain control PCR products with primers designed to analyze fragments containing *Hbb-y* and *Hbb-b2* owing to polymorphisms in the BAC clone #RP23-370E12. As a consequence, these fragments were not included in the locus-wide crosslinking frequency analysis (**Fig. 1**). We carried out all animal experiments according to institutional and national guidelines (Committee on Experiments with Laboratory Animals (DEC-Consult); Ministry of Agriculture, Nature and Food Quality, The Hague, The Netherlands).

**Southern blotting.** We treated fetal liver cells (from 14.5-d.p.c. embryos) as described above (with indicated formaldehyde concentrations), but we omitted ligation and analyzed 10 μg of purified DNA by Southern blotting. We used the following probes: *Hbb-bh1*, a 255-bp *Hin*fI fragment, which hybridizes to a 2.7-kb *Hin*dIII *Hbb-bh1* fragment and *Hbb-bh0* (5.5 kb) and *Hbb-bh2* (6.4 kb) pseudogene fragments; *Hbb-b1*, a 700-bp *Hin*dIII/*Nco*I fragment, which hybridizes to a 1.0-kb *Hin*dIII fragment and a *Hbb-b2* (8.6 kb) fragment; HS3, 300-bp PCR fragment, which hybridizes to a 2.0-kb *Hin*dIII fragment; *Pou3f1*, 100-bp PCR fragment, which hybridizes to a 4.0-kb *Hin*dIII fragment.

**Cell culture.** We cultured I/11 cells as described previously[22,23]. Briefly, we maintained proliferating I/11 cells in StemPro-34 containing 2 units $ml^{-1}$ human recombinant erythropoietin, 100 ng $ml^{-1}$ murine recombinant SCF, $10^{-6}$ M dexamethasone and 40 ng $ml^{-1}$ insulin-like growth factor. We expanded cells by daily partial medium changes and addition of fresh factors, keeping cell density between $1.5 \times 10^6$ and $4 \times 10^6$ cells per ml. To induce differentiation, we removed continuously proliferating I/11 cells from the culture, washed them twice in phosphate-buffered saline and seeded them at $2–3 \times 10^6$ cells per ml in differentiation medium containing 10 units $ml^{-1}$ erythropoietin, $4 \times 10^{-4}$ IE insulin per ml, the dexamethasone antagonist ZK-112993 ($3 \times 10^{-6}$ M) and 1 mg $ml^{-1}$ iron-saturated human transferrin. We maintained differentiating erythroblasts at densities of $2–6 \times 10^6$ cells per ml. For 3C analysis of differentiating I/11 cells, we fixed cells with formaldehyde 40 h after induction and processed them as described above.

**URLs.** Ensembl Genome Browser is available at http://www.ensembl.org. MRC gene service is available at http://www.hgmp.mrc.ac.uk.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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