Chapter 4

Structural dynamics of a nuclear compartment of *cis*-regulatory DNA elements and β-globin genes during erythroid differentiation and development.

Structural dynamics of a nuclear compartment of *cis*-regulatory DNA elements and β-globin genes during erythroid differentiation and development.

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Summary

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, and may be achieved by spatial clustering of chromatin-associated binding sites for such factors [75, 108, 152, 272, 273]. Here we analyse the structure of an erythroid-specific spatial cluster of *cis*-regulatory elements and active β-globin genes, the Active Chromatin Hub (ACH) [190], at different stages of development and in erythroid progenitors. We show, in mouse and man, that a core ACH is developmentally conserved and consists of the hypersensitive sites (HS1-6) 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 [19]. In murine erythroid progenitors that are committed to, but do not yet express globin, only the interactions between 5'HS-60/-62, 3'HS1 and HS at the 5' side of the LCR are stably present. Upon induction of differentiation, these sites cluster with the rest of the LCR and the gene that gets 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.

Results/discussion

The mouse and human β-globin locus contain an upstream LCR and multiple β-like genes arranged from 5' to 3' in order of their developmental expression (figure 4.1A and 4.3A). In addition there are several distal hypersensitive sites (HS), including a downstream 3'HS1 (approx. 20kb 3' of the β genes) and two upstream HS, ~60 kb (mouse) and ~110 kb (human) away from the genes [164]. The loci are embedded in an olfactory receptor gene cluster that is inactive in erythroid cells [162]. To investigate the spatial organisation of β-globin gene loci in mouse and man during development and erythroid differentiation, we applied chromosome conformation capture (3C) technology (see Methods [190, 229]). 3C-technology involves quantitative PCR-analysis of cross-linking 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 due to preferential restriction enzyme digestion of one site over the other (figure 4.1D-G; for other controls see Methods and [190]).

First, we determined the spatial organisation of the murine β-globin locus in primitive erythroid cells present in 10.5 dpc embryonic blood, that predominantly express the embryonic εy and βh1 globin genes [217]. Cross-linking frequencies were determined for 66 pairs of *HindIII* restriction fragments, spread over ~170 kb of DNA encompassing the murine β-globin gene cluster. The 3C-measurements indicate a basic structural organisation in primitive cells very similar to that observed previously in definitive blood cells isolated from 14.5 dpc fetal liver [190]. This is best illustrated by comparing the locus-wide cross-linking frequencies of a restriction fragment that contains HS4-5 of the LCR. Two peaks of high cross-linking 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 (figure 4.1B). Significantly lower cross-linking frequencies were found with fragments in between, suggesting that the LCR interacts with these distal HS through looping. The same interactions were observed in definitive blood cells that exclusively express the adult βmajor and βminor globin genes [217], where βmajor is also found in close proximity (figure 4.1B, and see below). In contrast, in non-expressing brain cells HS4-5 shows no peaks of interaction with distal DNA fragments, suggesting a linear conformation of the transcriptionally inactive locus [190]. Similar

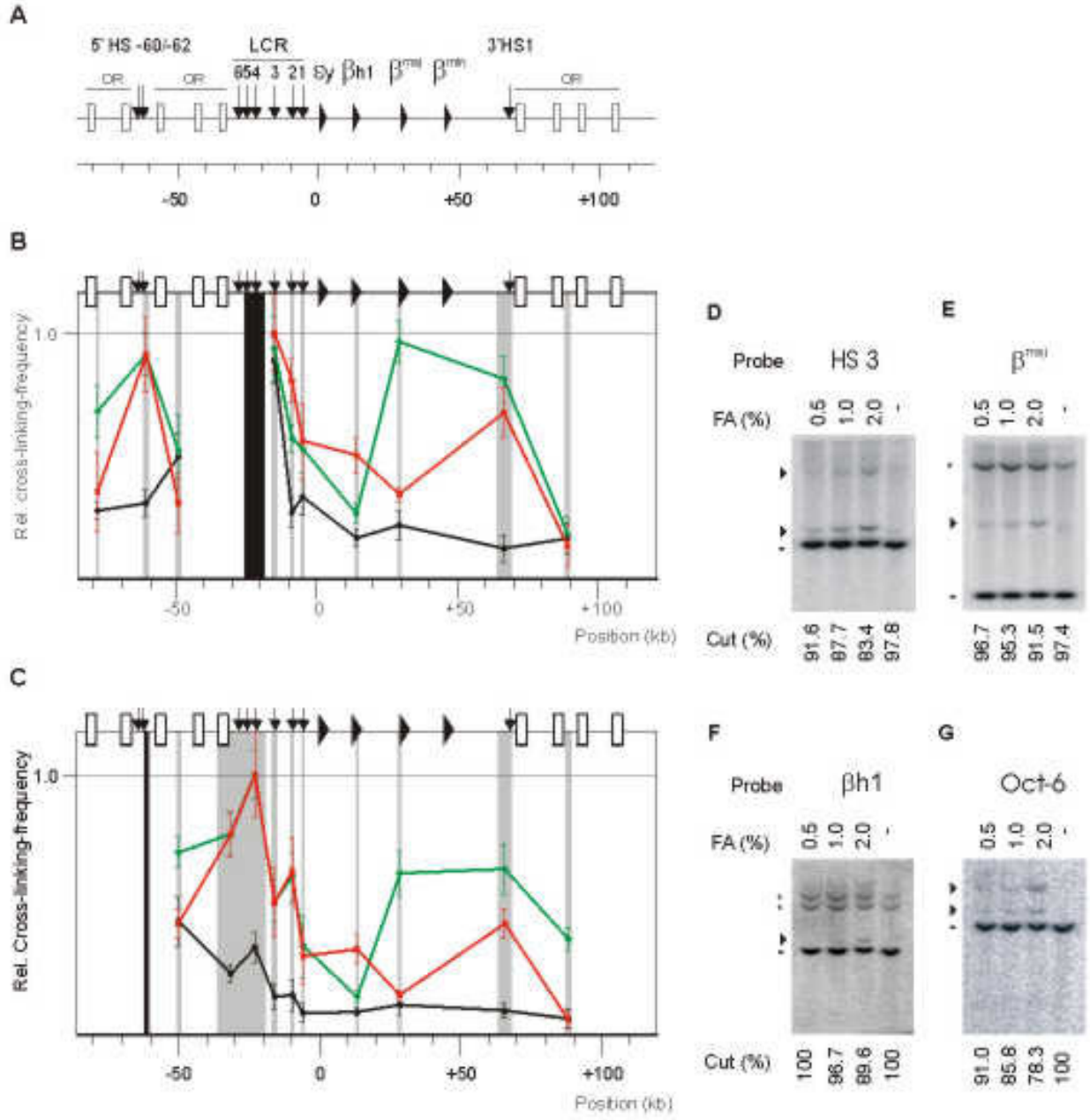

Figure 4.1. Spatial organization of the murine β-globin locus.

Schematic presentation of the mouse locus (A). Arrows depict the individual HSs, globin genes are indicated by triangles, and boxes indicate the olfactory receptor (OR) genes. Erythroid-specific and developmentally stable clustering of *cis*-regulatory elements (B-C). Relative cross-linking frequencies observed in primitive erythrocytes are shown in red, definitive erythrocytes in green, and non-expressing brain in black. Grey shading indicates position and size of the analysed fragments, while black shading represents the 'fixed' fragments HS 4-5 (B) and 5'HS –60/-62 (C). Within each graph, the highest cross-linking frequency value was set to one. The x-axis shows position in the locus.

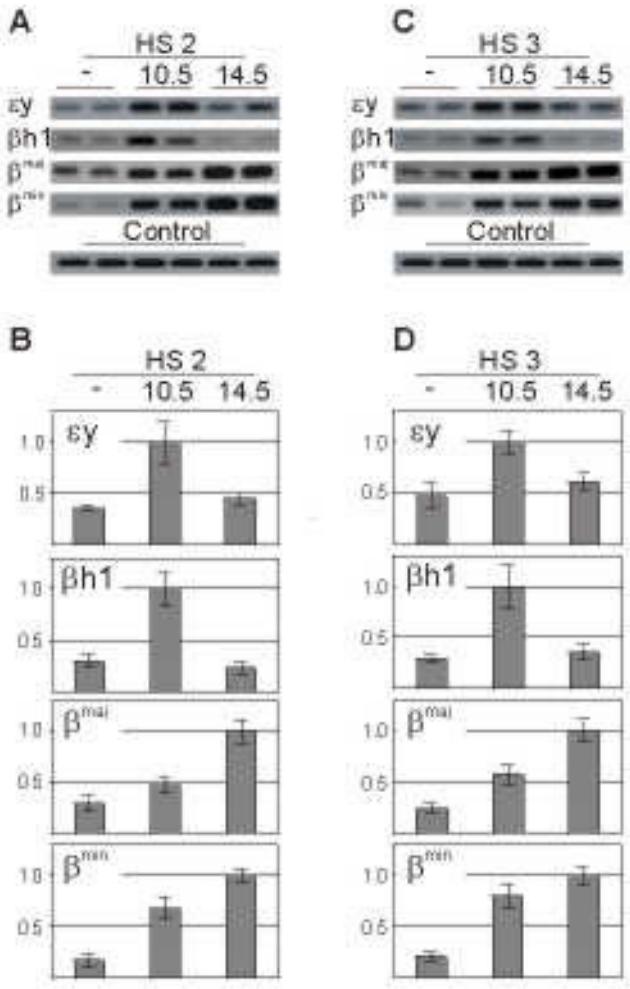

Figure 4.2. A Developmental switch occurs in contacts between individual β-globin genes and the core ACH of the murine β-globin locus.

Cross-linking frequencies of HS2 and the β-globin genes were measured (A-B). An example of PCR amplified ligation products is shown on 2% agarose gel (A), as well as the quantified data of all experiments (at least five, in duplo, per primer set) (B). (C-D) Identical to (A) and (B), but now for HS3 and the β-globin genes. Standard-error-of-mean is indicated. Non-expressing brain is depicted by '-', primitive erythrocytes by '10.5', and definitive erythrocytes by '14.5'. Control is PCR-amplified ligation product of two restriction fragments in the XPB locus (see Methods). Cross-linking frequencies shown in *b* and *d* are not corrected for PCR amplification efficiency and therefore only signals obtained with the same primer set can be compared.

results were obtained when analysing the locus-wide cross-linking frequencies of fragments carrying 5'HS-60/-62 (figure 4.1C) and other HS (data not shown): interactions among the *cis*-regulatory elements of the β-globin locus were found to be conserved between primitive and definitive erythroid cells. We conclude that the *cis*-regulatory elements of the murine 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 HS at –60 kb, all HS of the LCR and 3'HS1.

(Figure 4.1 continued). Standard-error-of-mean is indicated. Southern blots show that, in definitive erythrocytes digestion efficiency of cross-linked chromatin depends on formaldehyde concentration and is comparable between a hypersensitive site in the LCR (D), a transcribed gene within the locus (E), a non-expressed gene within the locus (F) and a non-expressed gene on a different chromosome (chr. 4) (G). Percentage formaldehyde cross-linking is shown at the top of each blot (- depicts genomic DNA not treated with formaldehyde), while the yield of specifically cut fragments is shown (percentages) at the bottom. Arrowheads depict partial digests and asterisks cross-hybridisation signals with other genes (see Methods).

The main differences in conformation between the two expressing cell types appear to be confined to interactions between the globin genes and the regulatory DNA elements. This is confirmed by measuring cross-linking frequencies with HS2 and HS3 of the LCR, two sites previously shown to be the most prominent transcriptional activating elements [15, 177, 180, 186, 187, 218]. The embryonic globin genes εy and βh1 were found to interact frequently with these elements in primitive erythroid cells, whereas in definitive red blood cells interaction frequencies between these sites dropped to levels similar to what was observed in the inactive brain (figure 4.2A-D). The opposite was seen for the adult βmajor and βminor genes, which interacted most frequently with HS2 and HS3 in definitive erythroid cells. Cross-linking frequencies between these sites in 10.5 dpc embryonic blood were not as low as in brain, probably due the fact that βmajor and βminor are already transcriptionally active at this stage, albeit at less than 10% of the levels observed in definitive cells. 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 βminor and 3'HS1, which is full of repetitive sequences, acts as a rigid region [190]. These data demonstrate 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.

To further investigate the significance of our findings, we analysed the conformation of the human β-globin locus at different stages of development. The mouse and human β-globin gene loci show a high degree of nucleotide sequence conservation, particularly at regions implicated in gene regulation [162, 274]. We made use of transgenic mice carrying a single copy of a 185 kb PAC (figure 4.3A) spanning the human β-globin locus that displayed a normal expression pattern [199, 232] (Patrinos, in prep.). Although large, this PAC does not include the human equivalent of the murine 5'HS-60/-62, which is located ~110 kb upstream of the human globin genes [162, 164]. We analysed the conformation of the transgenic human globin locus in 10.5 dpc embryonic blood, 14.5 dpc fetal liver and 14.5 dpc fetal brain, measuring almost all of the 120 site pairs that can be formed between the 16 *EcoRI* fragments that were selected for analysis. The locus-wide cross-linking frequencies of a fragment corresponding to 3'HS1 illustrate that also the transgenic human locus forms a core ACH, consisting of the 3'HS1 and the HS of the LCR, that is conserved in primitive and definitive erythroid cells (figure 4.3B). The structural changes we observed primarily concerned the position of the genes relative to this core ACH, correlating with transcriptional activity. Thus, the embryonic ε and the two γ genes most frequently interact with HS2-4 (figure 4.3C-E) and 3'HS1 (figure 4.3B) in primitive erythroid cells and the adult β gene primarily contacts the ACH in definitive cells (figure 4.3C-E). Identical results were found for a *HindIII* 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 dpc fetal liver cells (data not shown). It is interesting to also note the decreased cross-linking frequency of HS5 in the definitive cells as we have recently shown that this element has LCR blocking activity in primitive but not definitive erythroid cells [35]. We conclude that the overall spatial organisation of the β-globin gene cluster is conserved from mouse to man.

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. If exposed to physiologically relevant stimuli, I/11 cells synchronously undergo the normal *in vivo* differentiation program to mature terminally into enucleated erythrocytes [279, 280]. As expected, in differentiating I/11 cells that actively transcribe the adult β-like globin genes the locus adopts a spatial organisation very similar to what we observed previously in definitive erythroid cells isolated from fetal livers [190] (figure 4.4). However, in uninduced

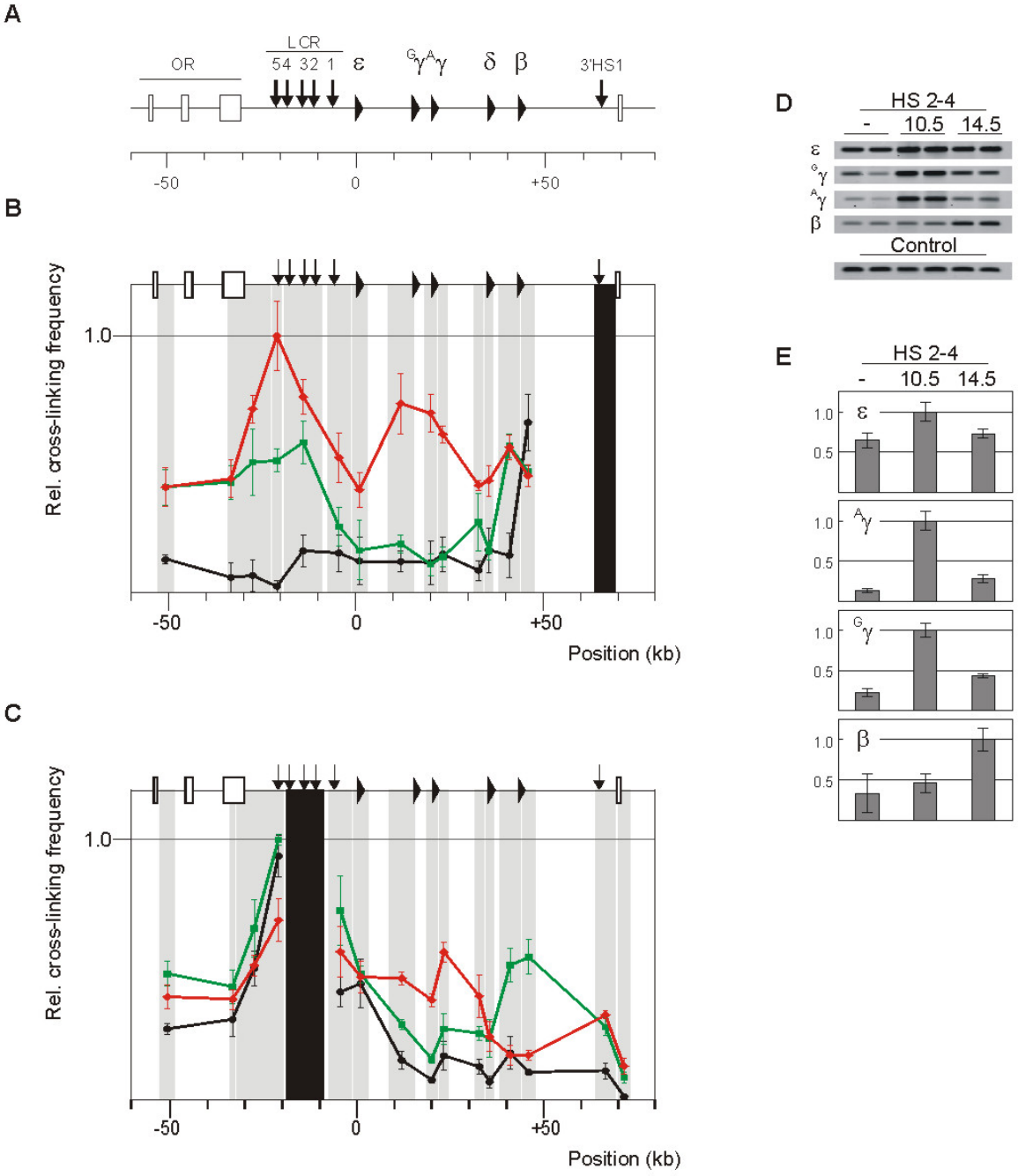

Figure 4.3. Spatial organization of the human β-globin locus.

Controls, symbols, color patterns and numbering are as in figure 1 and 2. Schematic presentation of the human locus (A). Locus-wide cross-linking frequencies of a 3'HS1 fragment show erythroid cell specific clustering with the LCR throughout development (B). Developmental switching in contacts of the LCR between the different β-globin genes as shown by locus-wide cross-linking frequencies of HS2-4 (C). The contacts between the HS2-4 of the LCR and individual β-globin genes alter during development in erythroid cells, as shown (D) by an example on agarose gel and (E) quantified data (at least five experiments in duplo per primer set).

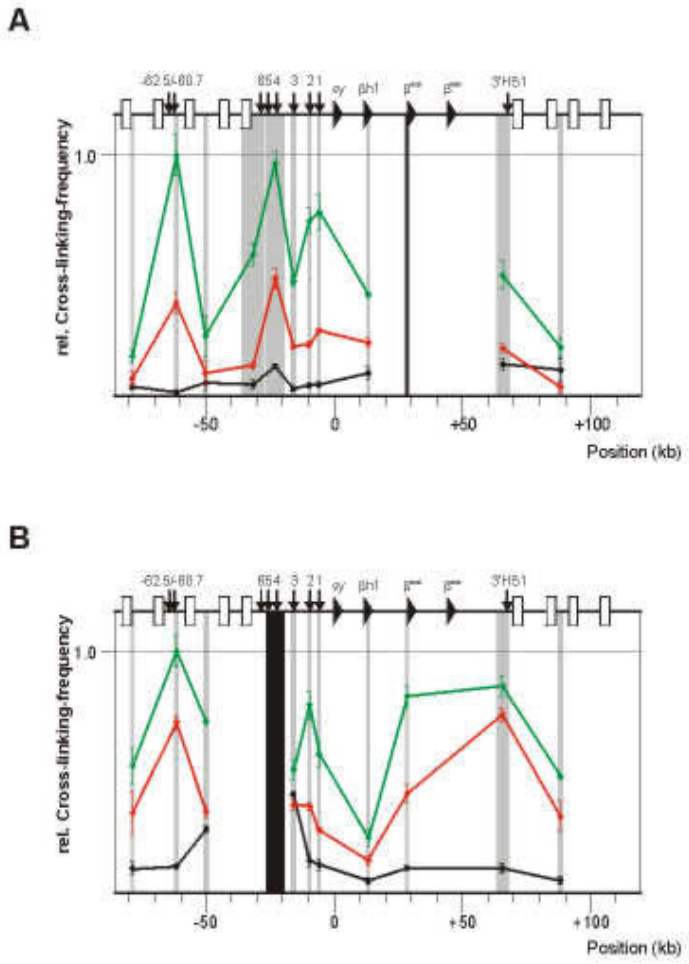

Figure 4.4. Spatial organization of the murine β-globin locus in erythroid progenitors.

Controls, symbols and numbering are as in figure 1, black lines represent brain, red lines proliferating l/11 erythroid progenitor cells and green differentiated l/11 cells. Locus-wide cross-linking frequencies of βmajor (A). Locus wide cross-linking frequencies of HS4-5 (B). Note that only the interactions among HS4-5, HS-60/-62 and 3'HS1 are already fully established in non-expressing progenitor cells.

proliferating l/11 cells that do not yet express the β-globin genes, a different structure is observed. Locus-wide cross-linking frequencies of a fragment corresponding to the βmajor gene were found to be reduced compared to those observed in erythroid cells expressing the gene (figure 4.4A). However, the structure of the locus poised for transcription is clearly different from that of the inactive locus in brain cells. This structure is better resolved by looking at the locus-wide cross-linking frequencies of the restriction fragment that contains HS 4-5 of the LCR. Two peaks of high cross-linking frequency with this fragment stand out in erythroid progenitor cells: one with 5'HS-60/-62 and another with 3'HS1 (figure 4.4B). 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 strongly reduced in progenitor cells (figure 4.4A-B, 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 (HS-60/-62 and 3'HS1) and HS at the 5' side of the LCR (HS4,5 or 6, we currently cannot say which of these HS is responsible for direct interaction). Upon induction of differentiation, clustering with the active genes and the complete LCR is established and the β-globin genes are being expressed (figure 4.5).

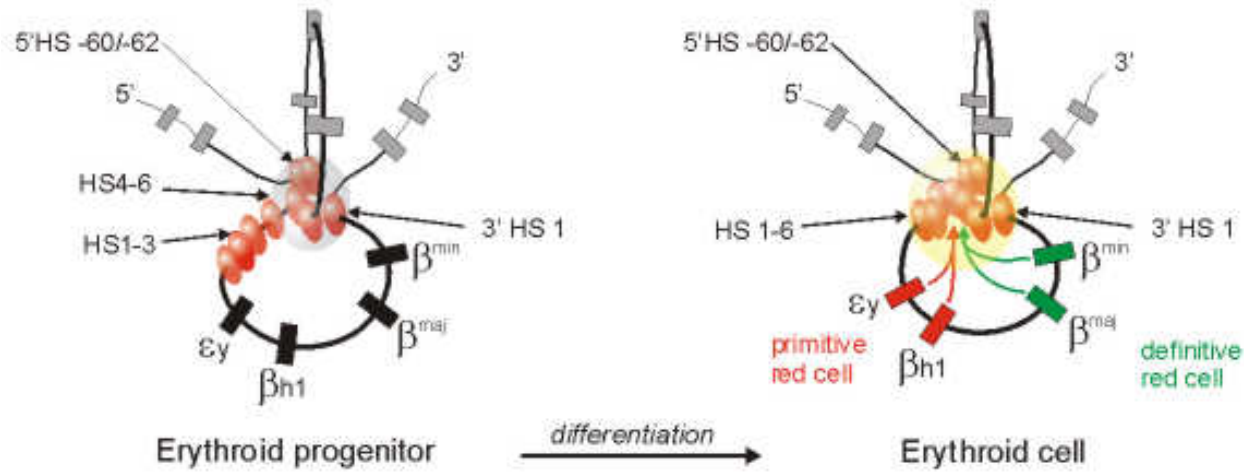

Figure 4.5. *Cis*-regulatory elements of the β-globin locus create a nuclear compartment dedicated to RNA polymerase II transcription: the Active Chromatin Hub.

2D-presentation of 3-dimensional interactions that occur between regulatory DNA elements 130 kb apart (red ovals) and β-globin genes (active: red and green rectangles; inactive: black) in erythroid progenitors (left) and differentiated primitive and definitive erythroid cells (right). In erythroid progenitors not expressing globin, a substructure (grey sphere) is present which is formed through interactions between the upstream 5'HS-60/-62, the downstream 3'HS1 and HS at the 5' side of the LCR (HS4-6; we currently cannot say which of these HS 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 sub-structure 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 depicted by the arrows. Inactive globin and olfactory receptor genes (grey squares) loop out.

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 (figure 4.5). A sub-structure is already present in erythroid progenitors that do not express globin, and it is worth noting that the three sites involved in this structure all bind CTCF [39]. It should be noted that 3'HS1 and 5'HS-60/-62 are dispensable for normal globin gene expression in transgenic mice [35, 199], suggesting 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 as a consequence 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 [108]. No pol 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 [110, 275]. 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 [111, 112] 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).

Isolation and formaldehyde fixation of primary cells, restriction enzyme digestion of cross-linked DNA in the nucleus, intramolecular ligation, reversal of cross-links, PCR-analysis of ligation products and calculation of relative cross-linking frequencies was done as described before [190, 229], with some modifications. Prior to fixation, cells obtained from embryonic blood (10.5dpc embryos), fetal liver and fetal brain (both 14.5dpc embryos) ($4x10^7$ cells per tissue) were forced 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 normalize ligation frequencies between globin site pairs to those detected between two restriction fragments (with the sites analysed 8.3 kb apart) in the XPB locus (instead of the previously used CalR locus [190]). XPB encodes a subunit of the basal transcription factor TFIIH, and we assumed that expression levels and spatial conformation of this gene are similar in all analysed tissues. To be able to compare signal intensities obtained with different primer sets in a quantitive manner, a control template is included 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 [190]). For the mouse β-globin locus we used a 214 kb BAC (#RP23-370E12, Ensembl Genome Browser, http://www.ensembl.org), and for the human β-globin locus we used a 185 kb PAC [232]. In addition, we used a 60-70 kb PAC containing the mouse XPB locus (PAC Clone #443-C18, MRC gene service, http://www.hgmp.mrc.ac.uk). We mixed either the mouse globin BAC or the human globin PAC with the XPB PAC at equimolar amounts. Subsequently, the mixes were digested and ligated as described [190]. We could not obtain control PCR products with primers designed to analyse fragments containing εy and βminor, due to polymorphisms in the BAC clone #RP23-370E12. As a consequence, these fragments were not included in the locus-wide cross-linking frequency analysis (see figure 4.1).

Southern blotting.

Fetal liver cells (14.5dpc embryos) were treated as above (with indicated formaldehyde concentrations), but ligation was omitted and 10 μg of purified DNA was analysed by southern blotting. The following probes were used: βh1, a 255 bp *Hinf*I fragment, hybridises to a 2.7 kb *Hind*III βh1 fragment and βh0 (5.5 kb) and βh2 (6.4 kb) psuedogene fragments; βmajor, a 700 bp *Hind*III/*Nco*I fragment, hybridises to a 1.0 kb *Hind*III fragment and a βminor (8.6 kb) fragment; HS3, 300 bp PCR fragment, hybridises to a 2.0 kb *Hind*III fragment; Oct-6, 100 bp PCR fragment, hybridises to a 4.0 kb *Hind*III fragment.

Cell culture.

I/11 cells were culture as described previously [279, 280]. Briefly, proliferating I/11 cells were maintained in StemPro-34™ containing 2 units/ml human recombinant erythropoietin, 100 ng/ml murine recombinant SCF, 10^{-6} M dexamethasone and 40 ng/ml insulin-like growth factor. Cells were expanded by daily partial medium changes and addition of fresh factors, keeping cell density between 1.5–4 $x10^6$ cells/ml. For induction of differentiation, continuously proliferating I/11 cells were removed from the culture, washed twice in PBS, and seeded at 2–3 $x10^6$ cells/ml in differentiation medium containing 10 units/ml Epo,
4 $x10^{-4}$ IE/ml Insulin, the Dex-antagonist ZK-112993 (3 $x10^{-6}$ M), and 1 mg/ml iron-saturated human transferrin. Differentiating erythroblasts were maintained at densities between 2-6 $x10^6$ cells/ml. For 3C-analysis of differentiating I/11 cells, cells were fixed with formaldehyde 40 hours after induction and processed as described above.

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