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The Higher Structure of Chromatin in the LCR of the β -Globin Locus Changes during Development

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Abstract

The β -globin locus control region (LCR) is able to enhance the expression of all globin genes throughout the course of development. However, the chromatin structure of the LCR at the different developmental stages is not well defined. We report DNase I and micrococcal nuclease hypersensitivity, chromatin immunoprecipitation analyses for histones H2A, H2B, H3, and H4, and 3C (chromatin conformation capture) assays of the normal and mutant β -globin loci, which demonstrate that nucleosomes at the DNase I hypersensitive sites of the LCR could be either depleted or retained depending on the stages of development. Furthermore, MNase sensitivity and 3C assays suggest that the LCR chromatin is more open in embryonic erythroblasts than in definitive erythroblasts at the primary- and secondary-structure levels; however, the LCR chromatin is packaged more tightly in embryonic erythroblasts than in definitive erythroblasts at the tertiary chromatin level. Our study provides the first evidence that the occupancy of nucleosomes at a DNase I hypersensitive site is a developmental stage-related event and that embryonic and adult cells possess distinct chromatin structures of the LCR.

Keywords

DNase I hypersensitivity; histones; nucleosomes; chromatin structure; 30-nm fiber

Introduction

Locus control regions (LCRs) are operationally defined by their ability to enhance the expression of linked genes to physiological levels in a tissue-specific and copy-number-dependent manner at ectopic chromatin sites.^{1–4} The first LCR, discovered in the human β -globin locus,⁵ spans about 16 kb and contains five DNase I hypersensitive sites (HSs 1–5). Each HS encompasses an array of binding motifs of erythroid-specific and ubiquitous transacting factors/cofactors, chromatin remodeling complexes, RNA polymerase II and other transcription factors. Recruitment of these factors to HSs in erythroid cells is thought to be the molecular basis of enhancer activity of the LCR and of the enhancer activity of individual HSs (mainly HSs 2–4). The intact LCR is capable of augmenting *in vivo* transcription of the cognate genes by 100- to 1000-fold. While the enhancer activity of the LCR is derived from the HS-recruited trans-acting factors, it is unknown how these factors make the LCR so powerful that it can function at a suppressive chromatin site.⁶ It has been speculated that the LCR has a

specific chromatin structure.⁷ The human β -globin LCR interacts either with the γ - or with the β -globin gene at a given time, although both the genes are expressed in the tissue.⁷ The fact that the LCR contains several HSs but only activates one gene at a time suggests that it may function as a single entity; this entity has been termed the LCR “holocomplex.”^{7,8} Results of HS deletions in transgenic mice are in agreement with this notion.^{8,9} Several models of the LCR holocomplex have been proposed on the basis of functional studies;^{8,10} however, the structure of the LCR holocomplex remains to be determined.

How the chromatin structure of the LCR modulates its enhancer activity is not well understood. Chromatin conformation could be divided into three hierarchical levels.¹¹ The “beads-on-a-string” would represent the primary structure. It is a 10-nm chromatin fiber consisting of an array of nucleosomes connected by linker DNAs. A typical nucleosome comprises 146 bp of DNA wrapped 1.65 times around an octamer containing two copies of histones H2A, H2B, H3, and H4. The folded nucleosomal array would define the secondary structure, which is often referred to as 30-nm fiber. The structure and *in vivo* existence of a 30-nm fiber are still being debated.¹² The crystal analysis of a tetranucleosome argues that the underlying structure of the 30-nm fiber is a zigzag two-start helix.¹³ The tertiary structure would refer to the folding of the 30-nm fiber. The structure beyond the 30-nm fiber is elusive. The establishment of long-distance interactions in the 30-nm fiber could be considered as one of the subtypes of such higher structure.

The LCR enhances the expression of all globin genes regardless of their developmental specificity, raising the possibility that it has a developmentally stable chromatin structure. On the other hand, the response of the LCR to mutations of the γ -globin gene promoter depends on the developmental stage of the erythroid cells.^{14–16} Differences between the LCR enhancer activity in embryonic and adult erythroblasts are also reflected in the finding that the LCR can drive the mouse heat shock protein 68 promoter in embryonic but not in adult erythroblasts.¹⁷ We speculate that the differences in LCR function between embryonic and adult stages of development originate from differences in LCR structure.

To investigate the possible structural changes of the LCR during development, we studied the chromatin structure of the wild-type LCR and subsequently evaluated the consequences when the LCR structure is perturbed by deletions of one of its DNase I hypersensitive sites (HS3). We found that nucleosomes at the HSs of the LCR are depleted in the embryonic stage of development, while they are retained at a regular level in definitive erythroid cells. The density of histone acetylation per histone at HSs is greater in the embryonic than in the adult erythroblast; the general accessibility of the LCR is greater in the embryonic stage compared to that of the adult erythroblasts; and the spatial proximity of the HSs of the LCR is closer in the embryonic stage relative to that of the adult erythroblasts. We propose that these differences result in distinct chromatin conformations at the 30-nm fiber level and that they are responsible for the different globin gene expression phenotypes of the LCR mutations.

Results

Nucleosome occupancy at HSs of the human and murine β -globin LCR is developmental stage specific

To study whether nucleosome occupancy of the β -globin LCR is independent of developmental stages, we measured the occurrence of histone H3 by ChIP in embryonic and adult erythroblasts of transgenic mice carrying a YAC construct containing the wild-type human β -globin locus. We found that the amount of histone H3 at all HSs (HSs 6, 5, 4, 3, 2, and 1) of the LCR was reduced in the yolk sac (Fig. 1a, right). These results were expected, as strong evidence supports the notion that the nucleosomes are usually depleted at an HS. However, we found that in the adult erythroblasts, histone H3 in the HSs was maintained at a level similar to that of the

amylase gene, which presumably has a regular nucleosome organization (Fig. 1a, left). The differences in histone H3 binding at the HSs between the embryonic and definitive stages were statistically significant. The levels of histone H3 in the intervening regions between HSs (between HSs 4 and 3, 3 and 2, 2 and 1, and HS1 and the ϵ gene) were similar to those of the control regardless of developmental stage (Fig. 1b).

The finding that histone H3 was not depleted at the HSs in definitive erythroblasts was unexpected. To ensure that the histone H3 measurements truly represented the level of nucleosomes, we estimated other three nucleosome components. Figure 1c shows the levels of histones H2A, H2B, and H4 in seven locations of the LCR in adult erythroblasts including four HSs and three intervening regions between the HSs. The results demonstrate that each type of histones is evenly distributed across the LCR from HS1 to HS4, and no meaningful differences were detected between the HS and the intervening regions.

We wondered whether the differences in nucleosome organization in the LCR at the two developmental stages were limited to the human β -globin locus transferred into the mouse genome or whether it also was a property of the endogenous murine β locus. To address this question, we estimated histone contents in the mouse globin locus. We found that all the five intervening regions of the LCR were occupied by normal amounts of histone H3 in either embryonic or adult erythroblasts (Fig. 1e). However, we found that while the HSs contained normal amounts of histone H3 in adult erythroblasts, a significantly reduced amount of histone H3 was detected at the HSs in embryonic erythroblasts (Fig. 1d). When the measurements were extended to histones H2A, H2B, or H4, we found that, similarly to the human LCR, each type of histones was evenly distributed across the HSs and the intervening regions in adult erythroblasts (Fig. 1f). These results indicate that in adult erythroblasts, both the endogenous mouse and the transgenic human LCRs are composed of a regular array of nucleosomes, which is not interrupted by the occurrences of the HSs. In contrast, in embryonic erythroblasts, the continuity of nucleosomes is interrupted by HSs.

The degree of acetylation per histone is greater in embryonic than in adult erythroblasts

To evaluate whether the level of histone acetylation in the LCR also changes during development, we measured the general acetylation of histone H3 in embryonic and definitive erythroblasts. We found that the level of histone acetylation in the LCR region was development independent. The HSs of the LCR were acetylated to a similar level both in embryonic and in adult erythroblasts (Fig. 2). However, it is worthwhile to point out that while the levels of histone acetylation at HSs of the LCR were similar at both stages, the degree of acetylation per histone was 1.5- to 2-fold greater in the embryonic than in the adult stage due to the depletion of nucleosomes in these sites in embryonic erythroblasts. On the other hand, as expected, histone acetylation of the globin genes correlated to the developmental stage related gene transcription. For instance, the β and δ genes were predominantly expressed in adult erythroblasts; correspondingly, two peaks of histone acetylation appeared in the β and δ gene regions (Fig. 4). Similarly, the peaks of histone acetylation in the yolk sac were located in the ϵ - and γ -globin genes.

Chromatin accessibility of the LCR is distinct in embryonic and adult erythroblasts

The findings described above suggest that the chromatin structure of the LCR may have different characteristics at different developmental stages. We employed a semiquantitative MNase sensitivity assay to evaluate this difference. At low concentrations, MNase preferentially cuts at the linker DNA; however, at high concentrations it preferentially cuts at open chromatin, and HSs created by DNase I or MNase perfectly overlap (Yin *et al.*, unpublished results). The assay was performed on the mouse endogenous β -globin locus. MNase digestions were carried out in the nuclei of the mouse yolk sac and adult spleen with

different enzyme concentrations and the remaining DNA was quantified by real-time PCR. The rapidity of the decrease in the amount of the DNA as enzyme concentrations increased reflected the nuclease sensitivity. To monitor the comparability of the digestion conditions between the two mouse tissues, we added human Jurkat cells into the yolk sac and spleen erythroblast samples prior to digestion.¹⁸ If the kinetics of nuclease cleavage of the external human genes is identical in two tissues, the data sets of digestion kinetics in the endogenous locus should also be comparable between these tissues. As shown in Fig. 3a and b, the kinetics of the human GAPDH (glyceraldehyde-3-phosphate dehydrogenase) gene and of a region next to the human 3'HS1 were identical in the yolk sac and adult spleen, suggesting that the experimental conditions in the two tissues were comparable. To further confirm the validity of this assay, we evaluated several endogenous regions whose sensitivities were predictable. We tested a region in mouse chromosome 1, which was in closed conformation, and its histones were barely acetylated (W. Yin and Q. Li, unpublished data). We found that the rapidity of DNA decrease of this region was slow, but identical in the two tissues (Fig. 3c). On the other hand, the mouse *εy* gene promoter was greatly susceptible to MNase digestion in the yolk sac, where the gene was expressed at a high level, whereas it was resistant in the adult erythroblasts of the spleen, where the gene was silenced (Fig. 3d), demonstrating that this assay can clearly distinguish the open conformation from the closed one. Collectively, the results demonstrated that the procedure can be employed to semiquantitatively detect chromatin openness and for comparison between different tissues.

The chromatin accessibility of five HSs and two intervening regions of the mouse LCR were evaluated in the yolk sac and spleen. As expected, the HSs (Fig. 3e–i) were more sensitive than the intervening regions (Fig. 3j and k). An unexpected feature was that in general, the LCR of the embryonic erythroblasts was more sensitive than that in the adult erythroblasts. This increased accessibility was characteristic of almost all the regions of the LCR. Another aspect was that each individual site was characterized by a specific accessibility. For example, among all HSs, HSs 2 and 3 were the most accessible. It should be pointed out that while the intervening regions of HS3/2 and HS2/1 (Fig. 3j and k) were relatively resistant to nuclease digestion, these regions still were more sensitive in comparison to the chromosome 1 region (Fig. 3c), suggesting that the intervening regions of the LCR were partially open in erythroid cells.

Collectively, the results suggest that as a whole, the LCR chromatin in embryonic erythroblasts is more sensitive than that in adult cells. This difference is small as measured by MNase sensitivity assay, but consistent. Differences in nuclease sensitivity reflect changes in chromatin conformation at the primary or secondary or both structure levels. Thus, we conclude that at the primary and/or secondary structural levels, the LCR in embryonic erythroblasts is more loosely packaged than that in adult erythroblasts.

Proximity between HSs of the LCR changes during development

It is unknown whether the developmental stage-related differences in nucleosome occupancy, the density of histone acetylation, and the degree of MNase sensitivity could also affect the higher chromatin structure of the LCR. We compared interaction frequencies between HSs of the human LCR in transgenic mice at different developmental stages by 3C assay. We found that the interaction frequency between HS1 and HS5 or HS2 and HS4 was greater in embryonic than in definitive erythroid cells (Fig. 4). The differences were statistically significant. These results suggest that the spatial proximity between the HSs of the LCR are closer in embryonic than in fetal erythroblasts.

The LCR responds differently to the HS mutations at different developmental stages

To investigate whether the distinct chromatin structure of the LCR has functional relevance, we studied two LCR mutants, which were previously produced in β YAC transgenic mice in

our laboratory.^{19–21} One is the HS3 core deletion (234 bp), which eliminates almost all known binding motifs of the HS site; the second is a deletion that removes a 2.3-kb segment encompassing the HS3 core and the flanking sequences. These two mutations had different impacts on the expression of the globin genes. The 2.3-kb HS deletion moderately reduced expression of the ϵ gene in the yolk sac and had no effect on expression of the γ and β genes in all stages of development. In contrast, the HS core deletion abolished expression of the ϵ gene in the yolk sac and disrupted expression of the γ gene in the fetal liver, and the β gene expression was reduced and subjected to the positions of integration.

We first estimated the effects of these deletions on HS formation at different developmental stages. Figure 5B, a–d was the wild-type control showing the formation of HSs 1–5 in embryonic and in fetal erythroblasts. We found that the 2.3-kb HS3 deletion did not disrupt the formation of these HSs in embryonic and fetal erythroblasts (Fig. 5B, i–l). However, the HS core deletion disrupted the formation of HSs 1–5 in the fetal liver (Fig. 5B, e and f), but did not in the yolk sac (Fig. 5B, g and h). These results suggest that while the different HS3 mutations differently affect gene expression, they also differently alter the conformation of the LCR.

3C analysis showed that in the yolk sac, the interaction frequency between HS5 and HS1 or HS4 and HS2 was not affected by either the HS3 or the HS3 core deletion (Fig. 5A-a), while in definitive erythroblasts of the fetal liver, the HS3 core deletion decreased the interaction frequency. In contrast, the 2.3-kb HS3 deletion increased the interaction frequencies between HS4 and HS1 and HS4 and HS2 (Fig. 5A-b). The increase apparently reflected the shortened distance between these HSs caused by the 2.3-kb deletion.

We evaluated the effects of the HS3 deletions on histone acetylation at different stages of development. The level of histone H3 acetylation was not affected by the HS3 core or the 2.3-kb deletion in embryonic erythroblasts (Fig. 5C). However, in adult erythroblasts, as reported previously,¹⁰ the HS3 core deletion disrupted histone acetylation of the LCR, while the 2.3-kb HS deletion did not. Thus, in the embryonic stage, neither the 2.3-kb nor the core HS3 deletion affects histone acetylation, whereas in the fetal and adult stages, histone acetylation was disrupted by the core HS3 deletion but not by the 2.3-kb HS3 deletion.

Finally, we asked if the HS3 deletions could affect the nucleosome occupancy of the LCR. We found that the 2.3-kb HS3 or HS3 core deletions did not change the occupancies of histones H3 or H4 in the HSs and in the intervening regions between HSs in adult erythroblasts (Fig. 5D, a and b). In embryonic erythroblasts, the histone depletion was also not affected either by the HS core or by the 2.3-kb HS deletion (data not shown). Thus, the status of histone occupancy of the LCR cannot be altered by these HS3 mutations either in embryonic or in adult erythroblasts.

Together, our results suggest that the two different HS3 deletions discriminately influence the LCR structure, which coincides with the special effects of these mutants on the expression of the globin genes.

Discussion

This study demonstrates that the wild-type β -globin LCR carries distinct chromatin structures at different stages of development either in the transgenic or in the endogenous settings. The different properties include histone occupancy and density of histone acetylation at HSs, general chromatin accessibility, and the spatial proximity between HSs. Studies on the two types of HS3 deletions suggest that these mutations, which differently affect the expression of the globin genes, also differently alter the conformation of the LCR at a different stage of

development. Hence, the developmental stage-related differences in chromatin structure may have functional consequence.

A widely accepted notion is that the structure and function of an LCR are unchanged during development and differentiation. This notion is supported by several observations. For example, the HSs of the β -globin LCR are indistinguishably formed in embryonic and adult erythroid cells, and the LCR is able to enhance the expression of the different cognate globin genes throughout the entire course of development.²² However, this notion has been challenged by functional studies that showed that the β -globin LCR seems to behave differently in different developmental stages. As mentioned earlier, the LCR could drive the mouse heat shock protein 68 promoter in embryonic but not in adult erythroblasts.¹⁷ This finding could be explained if the enhancer activity of the LCR is more powerful in embryonic than in adult erythroblasts. This notion is supported by additional observations that demonstrate that when an essential promoter element (CACCC, CCAAT, or TATA) of the γ -globin gene is disrupted, the expression of this gene is not affected in embryonic erythroid cells, but it is abolished in adult erythroid cells.^{14–16} As the LCR is able to drive deficient γ promoter to high levels of expression at the embryonic stage, but cannot do so in the adult stage, it seems likely that the enhancer activity of the LCR is more powerful in the embryonic compared to the adult erythroid cells. It is, however, still unknown whether these functional differences associate with changes in the conformational structure of the LCR.

One approach used for delineation of the relationships between structure and function of the LCR is to perturb the LCR structure and evaluate the functional consequences of the structural changes. Each HS of the LCR has been studied by deletion mutations in transgenic mice carrying the human β -locus, and the functional consequence of these mutations have been reported by several laboratories.^{6,8,9,19,20,23–26} The phenotypes of HS deletions of the endogenous murine β -globin locus have also been defined.^{24,25,27,28} Results of these studies are summarized in Table 1. In general, the severity of an HS deletion in the LCR inversely correlates with the size of the deletion in the setting of human β -globin transgenes. Thus, an HS core deletion usually results in a more severe perturbation on globin gene expression in comparison with larger deletion of the same HS. In addition, the effects are more profound in adult than in embryonic erythroblasts. These conclusions, however, are not consistent with the results obtained in the endogenous mouse β -globin locus setting. Thus, there were no significant differences between the phenotypes of a 0.33-kb deletion of the murine HS2 core and of a 1.1-kb deletion of the murine HS2, both done by homologous recombination in the endogenous mouse β -globin locus.^{25,28} It is worthwhile to note that similar differences between the phenotypes of HS mutations in the transgenic and endogenous loci have been reported for the α -globin locus. The element HS-40 of the human α -globin locus accounts for the majority of enhancer activity of the upstream regulatory region²⁹ of that locus. Removal of mouse HS-26, which is equivalent to the human HS-40, has only moderate effects on expression of the mouse α -globin genes.³⁰ In contrast, deletion of the HS-40 in a mouse humanized³¹ in the α -globin locus, in which the mouse α locus has been replaced with its human counterpart, results in abolishment of the α -globin gene expression, thus mimicking the phenotype of HS-40 deletion in patients with α -thalassemia.³¹ The differences in the effect of deletions in the endogenous and transgenic β -globin loci could be resolved by reproducing the deletions in mice carrying a humanized β -globin locus.

The link between chromatin structure and function of the LCR could become perceptible if we assume that the enhancer activity of the LCR associates with the sum of trans-activating factors recruited by each HS, and the final concentration is influenced by relative distances between the HSs. It has been proposed that the chromatin of the β -globin locus is seemingly decondensed to the 30-nm fiber level in erythroid cells.³² The 30-nm fiber of the LCR is bent to fit into the nucleus, and this forces the HSs to come closer to each other (Fig. 6a and b, left). Histones at

the HSs are acetylated, resulting in an increase in the flexibility of chromatin,³³ which would further bring the HSs closer to each other (Fig. 6a and b, right). Since the degree of acetylation per histone is greater in the embryonic than in the adult stage, the LCR chromatin could be folded into a tighter structure in embryonic than in adult erythroblasts (compare a with b, Fig. 6). This theoretical consideration is supported by the experimental results, which demonstrated that the spatial proximity between HSs 5 and 1 or 4 and 2 of the LCR is greater in embryonic erythroblasts compared to the spatial proximity of the same HSs in the definitive erythroblasts (Fig. 4). This structure could explain the observation that the enhancer activity of the LCR would be stronger in embryonic than in adult erythroblasts. This proposed structure predicts that the effect of a HS deletion on the expression of the globin genes would be a deletion size-dependent phenomenon (compare c with d, Fig. 6). This prediction is supported by the fact that the HS3 core deletion has more profound effects on globin gene expression than the 2.3-kb HS3 deletion.

It is broadly noted, but with unclear reasons, that the phenotypes of the HS2 deletion in the endogenous mouse and transgenic human β -globin loci were strikingly different. The model proposed here might provide a partial answer. This model predicts that changes in the distance between HSs will alter the strength of enhancer activity of the LCR. The distances between HS2 and HS3 and between HS3 and HS4 are different in the mouse and human β -globin loci,^{34,35} and the sizes of deletions used in different laboratories were not identical. Based on the model, thus, one should not anticipate an identical phenotype resulting from the HS2 deletion in the mouse and human loci.

Materials and Methods

Transgenic mouse models

The production of transgenic mice carrying the wild-type, 2.3-kb HS3, or 234-bp HS3 core deletion β YAC constructs was described previously.^{19,20} Eleven days postcoitum (dpc), yolk sacs or 14 dpc fetal livers were collected from timed-breeding human β YAC transgenic mice according to the standard procedures. Adult spleens were prepared from 10- to 12-week-old β YAC transgenic mice 4 days after onset of phenylhydrazine-induced hemolytic anemia. More than 85% of cells in the single-cell suspensions of the yolk sac, fetal liver, or phenylhydrazine-treated spleen were erythroblasts as measured by benzene staining. The animal studies were carried out according to procedures approved by the Institutional Animal Care and Use Committee at the University of Washington.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assays were carried out as described previously.¹⁰ Rabbit polyclonal anti-acetyl-histone H3K9, 14 (06-599) was purchased from Millipore (Billerica, MA). Anti-histone H3, H4, H2A, and H2B (ab1791, ab31827, ab18255, and ab1790) antibodies were obtained from Abcam (Cambridge, MA). Immunoprecipitations (IPs) were performed at least three times. All data for ChIP were expressed as a ratio of the PCR readings of a given primer set in IP and input DNAs over an IP/input ratio of a control gene, and the standard deviation was calculated.

Micrococcal nuclease sensitivity assay

Micrococcal nuclease (MNase) was purchased from Worthington Biochemical (Lakewood, NJ). Nuclei preparation from the single-cell suspension and MNase digestion were performed as described,³⁶ with modifications. Mainly, Jurkat cells were added to the mouse yolk sac or spleen prior to MNase digestion. Instead of Southern blot hybridization, the digested DNA was quantitated by real-time PCR. The retained DNA amounts at five different MNase concentrations were compared to the undigested starting sample, respectively.

Quantitative PCR

Real-time quantitative PCR was performed on Opticon 2 (MJ Research, Watertown, MA) or LightCycler (Roche, Indianapolis, IN) PCR machines. PCR reactions were performed using SYBR Green master mix according to the manufacturer's instructions (QIAGEN, Valencia, CA). PCR primers were designed using Primer 3 software; primer sequences are available on request.

Chromosome conformation capture assay

We performed chromosome conformation capture (3C) assays according to the protocol kindly provided by de Laat with slight modifications. Briefly, the single-cell suspension was cross-linked with 1% (v/v) formaldehyde at room temperature for 10 min. The cross-linked DNA was digested overnight with HindIII restriction enzyme and DNA fragments were ligated with T4 ligase at 16 °C overnight (14–16 h). The ligated 3C DNA was purified by extraction with phenol/chloroform and precipitation with ethanol. The 3C products were quantitated by real-time PCR and a mix of 3C products of two BACs encompassing the human β -globin locus and the *Ercc* gene, respectively, as standard DNA. The ligation frequencies between the site pairs in the globin locus were expressed as percentage of that of the mouse *Ercc3* gene.^{37–39} All data points were generated from an average of three to five different experiments performed by different persons.

DNase I hypersensitivity assay

DNase I was purchased from Roche. Single-cell suspensions were prepared from 11 dpc yolk sac or 14 dpc fetal livers of human β YAC transgenic mice. Isolation of nuclei, DNase I digestion, and Southern blot hybridization were performed as described previously.¹⁰ The genomic DNA was digested with NdeI and hybridized by a 0.7-kb probe (coordinates: Ch 11: 5,265,816–5,265,106, hg16) to detect HSs 1, 2, and 3. Restriction enzyme MfeI and a 0.6-kb probe (5,264,760–5,264,168) were used for detection of HSs 3, 4, and 5.

Abbreviations used

LCR	locus control region
HS	hypersensitive site
ChIP	chromatin immunoprecipitation
MNase	micrococcal nuclease
3C	chromosome conformation capture.

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Fig. 1.

Occurrences of histones H2A, H2B, H3, and H4 in the human transgenic and the mouse endogenous β -globin loci. Bindings of histones H2A, H2B, H3, and H4 were measured by ChIP assay using the specific antibodies. (a) Comparison of histone H3 amounts at the HSs of the human β -globin LCR between adult and embryonic erythroblasts. The tested HSs are shown at the *x*-axis. (b) Comparison of histone H3 amounts at the four HS intervening regions of the human β -globin LCR between adult and embryonic erythroblasts. HS4/3 represents the region between HS4 and HS3, and so on. (c) Histone H2A, H2B, and H4 bindings at the HSs and the intervening regions of the human LCR in adult erythroblasts. The data columns in each group are arranged based on their genomic order from 5' to 3' related to the ϵ -globin gene. (d) Comparison of histone H3 amounts at the HSs of the mouse endogenous β -globin LCR between adult and embryonic erythroblasts. (e) Comparison of histone H3 amounts at the five HS intervening regions of the mouse endogenous β -globin LCR between adult and embryonic erythroblasts. (f) Histone H2A, H2B, and H4 bindings at the HSs and the intervening regions of the mouse endogenous LCR in adult erythroblasts. The data columns in each group are arranged based on their genomic order from 5' to 3' related to the $\epsilon\gamma$ -globin gene.

Fig. 2.

The profiles of histone H3 acetylation in the β -globin locus in embryonic and adult erythroblasts. The level of histone H3 at K9 and K14 acetylation in the β -globin locus was measured in embryonic and adult erythroblasts by ChIP assay. Enrichments were corrected by the *mAire* gene. The line drawing on the bottom shows the human β -globin locus. The globin genes are marked by their names. The ϵ , G_γ and A_γ genes are expressed in the yolk sac and early fetal liver of transgenic mice; the δ and β genes are expressed in fetal liver and adult spleen erythroblasts. The olfactory receptor genes are shown as ovals. The arrows indicate the DNase I hypersensitive site flanking the locus, which are constantly present in erythroid cells at all stages of development. The HSs at the promoters, which are associated with developmental stage-specific gene expression, are omitted. The LCR consists of HSs 1–5.

Fig. 3.

Comparison of MNase sensitivity of the mouse β -globin locus between embryonic and adult erythroblasts. Schematic illustration of the mouse β -globin locus is shown in the line drawing. The $\epsilon\gamma$ - and β^H1 -globin genes are expressed in embryonic erythroid cells, and the β^{maj} - and β^{min} -globin genes are expressed in definitive erythroid cells. The ovals represent the olfactory receptor genes. The arrows indicate DNase I hypersensitive sites. (a and b) The MNase digestion profiles of the GAPDH gene and 3' HS1 of human Jurkat cells, which were added into the yolk sac and the spleen prior to the MNase treatment. The y-axis represents the portion of the remaining DNA after treatment over the untreated DNA at each enzyme concentration. The x-axis represents different MNase concentrations from zero (untreated) to 30 units. (c to

k) The MNase digestion profiles at the gene promoters and various positions of the LCR of the endogenous mouse β -globin locus in embryonic and adult erythroid cells.

Fig. 4. Developmental-stage-associated changes in the proximity between HSs of the LCR. 3C assay was employed to estimate the proximity between HS5 and HS1 and between HS 4 and 2 in embryonic and fetal erythroblasts. The asterisks indicate that the difference between two different stages is statistically significant ($n=4$).

Fig. 5.

Effects of the HS3 core and 2.3-kb HS3 deletions on the chromatin structure of the LCR. (a) Comparison of the effects of Δ HS3c and Δ HS3 on the proximity between 5HS5 and 5HS1 and between 5HS4 and 5HS2 (a) in the yolk sac and (b) in the fetal liver. The error bars represent standard deviation. (b) Southern blot hybridization to determine HSs 1, 2, 4, and 5 of the LCR in the yolk sac and fetal liver in transgenic mice. HSs 4 and 5 were detected in the MfeI-digested DNA (upper panels) and HSs 1 and 2 were detected in the NdeI-digested DNA (lower panels). FL, fetal liver; YS, yolk sac; wild, wild-type β YAC; Δ HS3c, the β YAC harboring a 234-bp deletion containing the HS3 core; Δ HS3, the β YAC harboring a 2.3-kb deletion of HS3. (c) Comparison of the effects of the HS3 core and 2.3-kb HS3 deletions on histone acetylation in embryonic erythroblasts. See details in the legend to Fig. 2. (d) Comparison of histone binding in the human LCR between the wild-type, Δ HS3 and Δ HS3c β YACs in adult erythroblasts. (a) Histone H3; (b) histone H4. The error bars represent standard deviation.

Fig. 6.

The putative 30-nm fiber of the LCR in a simplified form of the two-start zigzag model. The purple balls represent nucleosome cores, and the green line indicates linkers between nucleosomes. The drawings represent a segment of the LCR encompassing HSs 4, 3, and 2, which is ~7.5 kb in length, with the nucleosome spacing ~200 bp. A long 30-nm fiber has to bend in order to be packaged into the nucleus. The positions of HSs are indicated in blue. Note that in embryonic erythroid cells the HSs are located in the linkers as histones at HSs are depleted (b). In contrast, the array of nucleosomes is uninterrupted in the LCR in adult erythroid cells (a) as the regular level of histones is retained at HSs (see the text). The red spheres represent trans-activating factors recruited by HSs with the highest protein concentration at the center. When the distance between HSs is short and the chromatin fiber bends, the protein spheres will overlap. Assuming that enhancer activity of the LCR is a function of the concentration of trans-acting factors recruited by HSs, the overlapping will result in a synergistic effect on the enhancer activity of the LCR because the overall concentration of trans-activating factors in the LCR is greater than the sum of those recruited by each HS. The overlapping will be augmented in erythroid cells as HSs of the LCR are heavily acetylated, leading to higher chromatin flexibility (depicted as the bends at HSs in the figures). The overlapping will be further increased in the embryonic stage as the degree of acetylation per histone protein is higher in embryonic than adult erythroid cells [compare (a) with (b)]. This model predicts that the enhancer activity of the LCR is determined not only by trans-acting factors recruited by HSs, but also the spatial distance between HSs and the flexibility of chromatin. (c and d) Ten and one nucleosomes were presumably removed by the 2.3-kb HS3 and the HS3 core deletions, respectively. Both the deletions eliminate a protein sphere. However, the reduction of the final concentration of trans-acting factors in the 2.3-kb HS3-deleted LCR is partially compensated for by the decrease in the distance between HSs 2 and 4 as 10 nucleosomes were eliminated by the deletion (c and d). Therefore, the overall effect of

the 2.3-kb deletion on enhancer activity of the LCR would be more moderate than that of the HS3 core deletion.

Effects of the HS deletions of the LCR on globin gene expression

Table 1

Species ^a	Deletion size (kb)	Expression assay ^b	Expression embryonic stage ^c	Expression in definitive (fetal) stage ^c	Expression in definitive (adult) stage ^c	Reference
ΔHS2 Human	1.9	RPA	ε: 55 γ: 67	γ: 50 β: 50	β: 50	1
Human	0.72	SPA	ε: 0–5 γ: 0–66	γ: 0–10 β: 0–40	β: 0–38	2
Human	0.38	RT-PCR	ε: 0–10	γ: 16–47	β: 1–32	3
Mouse	1.1	RT-PCR	εγ: 88–100 βH1: 84–100		β: 70	4
Mouse	0.33	RT-PCR	εγ: 100 βH1: 90–100		β: 75–80	5
ΔHS3 Human	2.3	RPA	ε: 26–36 γ: 120	γ: 90 β: 63–83	β: 100	1
Human	1.4	SPA	ε: 5 γ: 30	γ: 10 β: 20–50	β: 15–50	2
Human	0.23	RPA	ε: 2 γ: 72	γ: 0–12 β: 11–24	β: 23	6
Human	0.23	RT-PCR	ε: 9 γ: 0–39	γ: 0–42 β: 0–5	β: 16–33	7
Mouse	2.3	RT-PCR	εγ: 82–88 βH1: 88–92		β: 82	8
ΔHS4 Human	0.88	SPA	ε: 100 γ: 100	γ: 100 β: 100	β: 100	2
Human	0.28	RPA	ε: 76–97 γ: 128	γ: 39–46 β: 54–62	β: 35	9
Human	0.28	RT-PCR	ε: 2 γ: 2–4	γ: 25 β: 2–3	β: 12–21	7
Mouse	2.7	RT-PCR	εγ: 92 βH1: 100		β: 93	10

^aHuman, the human β-globin locus as a YAC or a 70-kb linked cosmid construct, in transgenic mice; mouse, the mouse endogenous β-globin locus with an HS deletion by homologous recombination.

^bSPA, S1 nuclease protection assay; RPA, RNase protection assay; RT-PCR, reverse transcription PCR.

^cExpression levels are shown as % of the wild-type locus. Some data in the table are an evaluation based on the information provided in figures or descriptions in the literature because no table was presented.

6, 8, 9, 19, 20, 24–28