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Cooperativeness of the Higher Chromatin Structure of the β-Globin Locus Revealed by the Deletion Mutations of DNase I Hypersensitive site 3 of the LCR

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Abstract

High-level transcription of the globin genes requires the enhancement by a distant element, the locus control region (LCR). Such long-range regulation *in vivo* involves spatial interaction between transcriptional elements, with intervening chromatin looping out. It has been proposed that the clustering of the HS sites of the LCR, the active globin genes, as well as the remote 5′ hypersensitive sites (HSs) (HS-60/-62 in mouse, HS-110 in human) and 3′HS1 forms a specific spatial chromatin structure, termed active chromatin hub (ACH). Here we report the effects of the HS3 deletions of the LCR on the spatial chromatin structure of the β-globin locus as revealed by the chromatin conformation capture (3C) technology. The small HS3 core deletion (0.23 kb), but not the large HS3 deletion (2.3 kb), disrupted the spatial interactions among all the HS sites of the LCR, the β-globin gene and 3′HS1. We have previously demonstrated that the large HS3 deletion barely impairs the structure of the LCR holocomplex, while the structure is significantly disrupted by the HS3 core deletion. Taken together, these results suggest that the formation of the ACH is dependent on a largely intact LCR structure. We propose that the ACH indeed is an extension of the LCR holocomplex.

Keywords

locus control region; chromatin conformation capture; DNase I hypersensitive site; globin; enhancer

Introduction

It has been recognized for a long time that chromatin is an active participant in transcription activation in higher eukaryotes.[1,2] Alteration of chromatin accessibility for transacting factors (open/closed chromatin) is a major step in regulating gene activity.[3] Recent advancements in histone modification studies suggest that the regulation of gene activity at the chromatin level is dependent not only on the accessibility, but also on the chromatin binding specificity for transacting factors. The different combinations of covalent modifications at the histone tails give rise to distinct binding possibilities for the transacting factors.[4,5]

In addition to accessibility and binding specificity, the spatial structure of the chromatin also regulates gene transcription. It is conceivable that like a protein, a 30 nm chromatin is folded up to form a 3D structure in the nucleus.[6,7] Because of the lack of appropriate techniques, the accurate tertiary structure of chromatin is unclear. However, the intensive studies in the globin

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locus in the past two decades have revealed that the chromatin tertiary structure is one of the major players in the *in vivo* regulation of globin gene expression. This view is supported by the two types of data: the holocomplex structure of the β-globin locus control region (βLCR),[8–10] and the active chromatin hub structure formed between the LCR, globin genes and the far upstream and downstream DNase I hypersensitive sites (HSs).[11,12]

It has been demonstrated that the LCR can only interact with one of the downstream genes at any given time, suggesting that the LCR functions as a single entity.[8] This hypothesis is reinforced by the deletion studies of individual HS sites in both knockout and transgenic mice.[13–18] The LCR is consisted of five HSs, spanning ~6 kb to 22 kb 5′ to the ε-globin gene. Each HS has a unique contribution to the globin gene transcription. When one HS, including its core and the flanking region, is deleted, the expression of the globin gene is reduced by ~20% to 30%. However, if only the HS core is deleted, the effect on globin gene expression is catastrophic. For instance, the deletion of the HS3 core resulted in abolishment of ε gene expression in the yolk sac and of γ gene expression in the fetal liver of the mutant transgenic mice. These results seem contradictory at first: how can a small HS core deletion yield profound effects, whereas a large deletion of a HS site, which includes the core, results in a mild phenotype. However, these different properties of the LCR can be well reconciled by hypothesizing that the LCR forms a specific tertiary chromatin structure, termed holocomplex.[8–10] In this model, an alteration in the 3D structure of the LCR may result in either an additive or synergistic effect on globin gene expression. Thus, the complex regulation of the globin locus is set on a specific tertiary chromatin structure.

Many multigene clusters contain a distal enhancer/LCR. The mechanism of long-range action of a distal enhancer is still unclear. Several models have been put forward to explain the distant effects. Among them, the looping model received the most support from studies using newly developed techniques, namely chromatin conformation capture (3C) and RNA tagging and recovery of associated protein (RNA Trap).[12,19,20] In the globin locus, the expressed globin genes are in close proximity to the LCR. Interactions of the LCR with the upstream HS site (HS-60/-62 in mouse, HS-110 in human) and downstream 3′HS1 can be consistently detected by the 3C technique in the erythroid tissues (fetal liver and yolk sac), as well as in progenitor cells.[11] These results have led to a model, termed the active chromatin hub (ACH).[11,12] A chromatin hub is created by looping HS-60/-62, 3′HS1, and the 5′ part of the LCR together. This higher chromatin structure is not associated with gene transcription. Upon differentiation, the rest HS sites of the LCR and the activated genes are looped into the hub, forming an active chromatin hub. Erythroid-specific transacting factors, such as EKLF,[21] GATA, and FOG[22] are essential for the establishment/stabilization of the ACH. A deletion of the β gene promoter or the HS3 site (1.2 kb) does not affect the ACH formation; however, the combination of these two deletions would disrupt the ACH structure.[23]

Previously, we studied the LCR holocompelx in the context of HS3 mutant βYAC transgenic mice.[10] In contrast to the mild effect of the large HS3 deletion (2.3 kb), the small HS3 core deletion (0.23 kb) had a profound impact on expression of the embryonic, fetal and adult globin genes. Moreover, the effects of the HS3 core deletion on the chromatin were not restricted to the HS and its neighboring regions, but were locus-wide.[10] The removal of the core element of HS3 disrupted the formation of all the remaining HS sites of the LCR and dramatically reduced the level of histone acetylation in the entire locus. Here we report that the higher chromatin structure remains intact in the β-globin cluster carrying the large HS3 deletion, but can be disrupted by the small HS3 core deletion of the LCR, as revealed by the 3C technology. These results suggest that the whole globin locus, including the upstream and downstream regions as well as the LCR, is organized into one specific tertiary chromatin structure with a quasi-equilibrium property. This chromatin structure will collapse when the basic conformation of the LCR is disrupted.

Results

The β-globin gene is in close proximity to the LCR in adult erythroblasts

In previous studies we demonstrated the dual nature, the additive and synergistic properties, of the LCR enhancer revealed by the 2.3 kb and the 0.23 kb deletions of HS3 in the context of the βYAC transgene.[10] To explain the dual properties of the LCR, we proposed that the LCR chromatin forms a cherry blossom-like 3D structure, which was barely affected by the 2.3 kb HS3 deletion. In contrast, the 0.23 kb HS3 core deletion resulted in a loose chromatin conformation, leading to a dramatic decrease in the enhancer strength of the LCR. To further delineate the functions of the LCR enhancer, we estimated the effects of these HS3 mutations on the long distance interaction between the LCR and the genes by 3C technology. Adult splenic erythroblasts prepared from transgenic mice were cross-linked with formaldehyde, and the fixed chromatin was restricted with HindIII. Re-ligation of the restriction fragments was performed at a low DNA concentration in order to facilitate the intramolecular ligation. Then, the cross-links were reversed and ligation products were quantitated by real-time PCR. A BAC containing the 185 kb human β-globin locus and a 230 kb BAC encompassing the Ercc3 gene were mixed in equal molar ratio, restricted with HindIII, and re-ligated. The mixture of the BAC DNAs served as both the positive control and the DNA standard in real-time PCR quantitation. The Ercc3 gene is a subunit of basal transcription factor TFIIH, and presumably it is expressed at a constant level.[12] The non-ligated samples were used as negative control.

We performed 3C analysis to estimate the proximity of the LCR to the expressed and non-expressed genes. HindIII cuts in between the hypersensitive sites of the LCR, producing 1.9 kb to 3.3 kb fragments and each HS site resides in one of these fragments. This feature facilitates the analysis of the spatial conformation of the individual HS sites in relation to the globin genes. Splenic erythroblasts highly express the adult β-globin genes whereas γ-globin gene expression is undetectable. When the β-globin gene was used as the anchor fragment, the yields of PCR products between the β gene and the HS sites were high (Figure 1(a)). Notably, all the five HindIII fragments containing the HS sites produced high yields. However, the products between the β gene and the region 5′ to the LCR (HS6, HS7 and the sequence between) were lower than those in the LCR region. In contrast, when the $^{G}\gamma$-globin gene was used as an anchor, the PCR products between the γ gene and the LCR were low, particularly, in the HS3 region (Figure 1(b)). In a reciprocal experiment, we digested the cross-linked chromatin with EcoRI. In this case HSs 2, 3, and 4 were located in one large fragment. Using this LCR fragment as the anchor, the product from the β-globin gene was two- to threefold higher than that from the γ-globin gene (data not shown). These results suggest that the expressing β-globin gene is in closer proximity to the LCR in comparison with the non-expressing γ-globin gene, though the latter is closer to the LCR based on the genomic distance.

The 0.23 kb HS3 core, but not a 2.3 kb HS3 deletion disrupts the interaction between the β-globin gene and the LCR

Figure 2 shows the effects of two different HS deletions on the interaction between the LCR and the β-globin gene. The 2.3 kb HS3 deletion in the βYAC construct had no effect on the interaction between the β-globin gene and all the HS sites of the LCR as estimated by 3C assay (comparing Figure 2(a) and Figure 1(a)). Since the HindIII fragment encompassing the HS3 site was deleted in the construct, we used a primer between the resultant fragment from the fusion of the two HS3 neighboring fragments for the 3C assay. Interestingly, this fragment retained a high PCR yield with the β-globin gene. The mild or no effect of the HS3 deletion on the interaction between the β-globin gene and the LCR is consistent with the fact that this mutation produced a mild phenotype in transgenic mice as evaluated by its influence on the HS formation, the histone acetylation and the β-globin gene expression in adult erythropoiesis.

In contrast to the 2.3 kb HS3 deletion, the 0.23 kb HS3 core deletion reduced the interaction between the β-globin gene and the LCR to a basal level (Figure 2(b)). Notably, this mutation dismissed not only the interaction between the HS3 and the β-globin gene, but also the interaction with all other four HS sites, suggesting that the HS sites of the LCR function as a unified entity. The lack of interaction between the β-globin gene and the LCR mirrors the low β-globin gene expression caused by the HS3 core deletion.

Effect of the HS3 core deletion spreads to the 3′ HS1 region

A DNase I hypersensitive site, designated as 3′HS1, was detected ~20 kb downstream of the β-globin gene in the endogenous human genome. This site was implicated in a higher chromatin structure, ACH, in erythroid cells. However, this HS site fails to be formed in the YAC or the cosmid human globin transgene in mice [10;,24]. To understand the role of 3′ HS1 in the regulation of globin gene expression, we estimated the interaction of this site with the LCR and the globin genes by 3C assay. Figure 3(a) shows that the 3′HS1 region yielded high PCR products with each the HS sites of the LCR, with HS5 being the highest. When using 5′HS5 as the anchor (Figure 3(b)), the PCR product of the 3′HS1 site was higher than the HindIII fragments between them, suggesting that 3′ HS1 and HS5 are in close proximity in adult erythroid cells. This spatial relationship between 3′HS1 and HS5 essentially remained unchanged when the 2.3 kb HS3 site was deleted (compared Figure 3(a) and (b) with (c) and (d)). In contrast, the 0.23 kb HS3 core deletion resulted in a two or more fold decrease of the PCR products of 3′HS1 with all HS sites of the LCR (Figure 3(e)). Similar results were obtained when the HS5 was used as the anchor in comparing the effects of the two HS3 deletion mutations (Figure 3(f)). These results suggest that the spatial relationship of 3′HS1 with the LCR is regulated by the LCR.

Discussion

It has been proposed that the β-globin locus forms an erythroid-specific spatial structure composed of *cis*-regulatory elements and active β-globin genes, termed the active chromatin hub.[11,12] A core ACH is developmentally conserved and consists of the hypersensitive sites of the LCR, the upstream 5′ HS and the downstream 3′HS1. The appropriate spatial configuration of the ACH requires multiple interactions between the LCR and the β-globin gene *in vivo*. The most prominent feature uncovered in our study is that the HS3 core deletion, but not the large HS3 deletion, disrupts not only its interaction with the β-globin gene, but also all other HS sites of the LCR as well as 3′HS1. Previously, we demonstrated that the large HS3 deletion barely perturbs the structure of the LCR holocomplex, while the structure is significantly disturbed by the HS3 core deletion.[10] Here, we show that the HS3 core deletion, which is 0.23 kb in length, is sufficient to disrupt the spatial conformation of the ACH, which spans more than 100 kb, suggesting that the formation of the ACH depends on a largely intact LCR structure. We propose that the β-globin locus ACH is a quasi-equilibrium chromatin structure (QECS) in erythroid cells. Cooperativeness of a minimum amount of *cis* elements is required for stabilizing the spatial conformation. When one of the critical components is absent, the QECS will collapse. This notion is consistent with the observations that the 1.2 kb HS3 deletion alone did not dramatically affect the HS formation of the LCR, histone acetylation, or long distance interaction in the globin locus; however, the combination of this mutant with the β gene promoter deletion had locus-wide impact, resulting in the phenotype similar to that of the HS3 core deletion.[23] Thus, it is likely that the formation of the QECS requires a mass action.[25]

A simple explanation for the reduction of PCR products between the β-globin gene and the HS sites caused by the HS3 core deletion is that the mutation damages the loop formation between the β-globin gene and the LCR. This interpretation, however, is at odds with the *in vivo* property

of the LCR. Despite its low level, the β-globin was expressed in this particular mutant transgenic line.[14] It has been documented that without the LCR enhancer as in transgenic mice carrying a LCR-less YAC construct, the expression of the β-globin gene would be undetectable (<0.1% of murine α mRNA).[26] Such a situation differs from that in the LCR knockout mice, where a residual expression of the β^{maj} gene (1–3%) can be detected.[27] Thus, the low PCR products in the HS3 core deletion measured by 3C assay do not necessarily imply that there is a lack of chromatin looping between the β-globin gene and the LCR, but rather that there is likely a reduced frequency of loop formation due to the mutation. From the reasoning given above, the 3C assay measures not only the proximity between the two points at a chromatin, but also the frequency of interaction between these two points. We have previously reported that the HS3 core deletion disrupts histone acetylation within the whole region from upstream of the LCR to downstream of the β gene, while the larger HS3 deletion only has a mild affect.[10] If loop formation is modulated by histone acetylation *via* its effect on chromatin flexibility, the lower level of histone acetylation in the HS3 core deleted β-globin locus may result in a reduction of the interaction frequency between the LCR and the β-globin gene.

It has been documented that 3′HS1 is not formed in transgenic mice carrying either YAC or cosmid human β-globin locus.[10,24] However, the interaction of this sequence with the LCR and globin genes can be detected by 3C assay in mice, suggesting that the formation of a hypersensitive site is not essential for the establishment of a spatial chromatin structure. The chromatin hub structure consisting of 3′HS1, HS-60/-62, and HS sites of the 5′ site of the LCR (5′ HSs 4, 5 and 6) is detected in mouse erythroid progenitors that are committed to but yet do not express β-globin.[11] After the induction of differentiation, the rest of the LCR and the genes will cluster with the chromatin hub and the hub is converted to an active status, termed active chromatin hub.[11] In this model 3′HS1 participates in the basic chromatin hub. It is unclear if the ACH is at a higher level of the regulatory hierarchy of globin gene activation or is at the same level as the LCR. Here, we show that the HS3 core deletion significantly damages the interaction of 3′HS1 and 5′HS5, suggesting the presence of a mutual influence between the ACH structure and LCR holocomplex. Since the formation and/or stability of the ACH are dependent on the intact tertiary chromatin structure of the LCR, it is possible that ACH is an extension of the LCR holocomplex.

The developmentally proper regulation of globin gene expression can be recapitulated in transgenic mice carrying the entire β-globin locus but lacking 3′ HS1 and/or the remote upstream HS sites.[28,29] Recently it has been reported that knockout of the far upstream region encompassing HS-60/-62 has no effect on chromatin opening and globin gene expression.[30] Thus, the functional relevance of the interaction between the upstream HS sites (HS-60/-62 in mouse and HS-110 in human) and the downstream 3′HS1 revealed by 3C assay remains unclear.[23] Since chromatin looping formation is a flexibility-dependent event,[31] the interactions of 3′HS1, HS5, and the upstream HS sites may reflect the preferential looping positions at the given chromatin flexibility.

Recently, the implication of the results from transgenic studies has been debated. A recent example is provided by the results of Fiering *et al.*[32] showing no difference in core *versus* larger deletions of the endogenous mouse HS2 sequences. These results contrast to the findings in transgenic lines with deletions of human HS2, in which deletion of the core sequences of HS2 had catastrophic effects on the globin phenotype.[9] It is unclear whether the strikingly different phenotypes reflect the species specificity or the difference between the natural *versus* ectopic chromosome locations of the deletions or functional differences between the murine and the human beta locus LCR. It should be mentioned here that differences between the murine and human regulatory elements have been well documented in the alpha locus where deletion of the upstream regulatory element HS-40 has minimal phenotypic effects in the mouse[33] while it produces a clear cut thalassemic phenotype in human.[34]

Materials and Methods

Materials

The production of transgenic mice carrying the wild-type, 2.3 kb HS3, or 234 bp HS3 deletion βYAC construct were described p.[13,14] Concentrated HindIII, EcoRI, and T4 DNA ligase were from New England BioLabs (Beverly, MA). QuantiTect SYBR Green PCR kit was from Qiagen (Valencia, CA). Real-time PCR was performed using a DNA Engine Opticon 2 (MJ Research, Waltham, MA).

Chromosome conformation capture (3C) assay

We performed 3C assays according to the protocol kindly provided by de Laat with slight modifications.[11,12] Single-cell spleen suspensions were prepared from 10–12 week-old βYAC transgenic mice four days after the onset of phenylhydrazine-induced hemolytic anemia. The cell suspension (~10^8 cells from one phenylhydrazine treated spleen) was diluted to 50 ml with RPMI 1640 medium. Splenic cells were cross-linked with 1% (v/v) formaldehyde at room temperature for 10 min. The reaction was quenched by adding glycine to a final concentration of 0.125 M. Cells were lysed in ice-cold lysis buffer (10 mM Tris (pH 8.0), 10 mM NaCl, 0.2% (v/v) NP 40) containing protease inhibitors. For one 3C reaction, approximately 5×10^6 to 7×10^6 of nuclei were re-suspended in 0.5 ml of restriction buffer containing 0.3% (w/v) SDS by incubation at 37 °C for 1 h with gentle shaking. Triton X-100 was then added to 1.8% (v/v), and the nuclei were further incubated for 1 h at 37 °C to sequester the SDS. The cross-linked DNA was digested overnight with the restriction enzyme (HindIII or EcoRI). Then, SDS was added to final concentration 1.6%, and the restriction enzyme was inactivated at 65 °C for 25 min. The reaction was further incubated at 37 °C for 1 h after dilution with 6.8 ml of ligation buffer (50 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, 25 mg/ml bovine serum albumin (BSA)), and Triton X-100 was added to 1%. DNA fragments were ligated with T4 ligase at 12 °C overnight (14–16 h). Cross-linking was reversed by adding proteinase K and incubating at 65 °C overnight. The DNA was concentrated by ethanol precipitation and resuspended in 0.5 ml of low TE buffer (1 mM Tris–HCl(pH 8.0), 0.05 mM EDTA). The samples were incubated with RNase A at 37 °C for 2 h, and DNA was purified by phenol extraction and ethanol precipitation.

PCR analysis of the ligation products

The ligation products were quantitated by real time PCR using SYBR green as the fluorescence dye. To correct the differences in ligation and PCR efficiency between the different sets of templates, we prepared a control template. Equimolar amounts of a 185 kb BAC (BAC Clone CTD2596M16; a kind gift from Dr D. Bodine) containing human β-globin locus and a 230 kb BAC containing the mouse Ercc3 locus (BAC Clone RP23-148C24; BACPAC Resources, CA) were mixed, then digested and ligated as described. The mix was used as the DNA quantity standard. The ligation frequencies between the globin site pairs were normalized to those detected between two restriction fragments in the mouse Ercc3 locus and expressed as percentage of the Ercc3 gene. Ercc3 encodes a subunit of the basal transcription factor TFIIH; presumably, the expression levels and the spatial conformation of this gene remain constant in all tissues.[12] The 5′ side of each restriction fragment was routinely used for designing primers unless this coincided with repetitive DNA sequences. Primer sequences are available upon request. All data points were generated from an average of three to five different experiments performed by different persons.

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Abbreviations

LCR	locus control region
ACH	active chromatin hub
HS	hypersensitive site
3C	chromatin conformation capture
QECS	quasi-equilibrium chromatin structure

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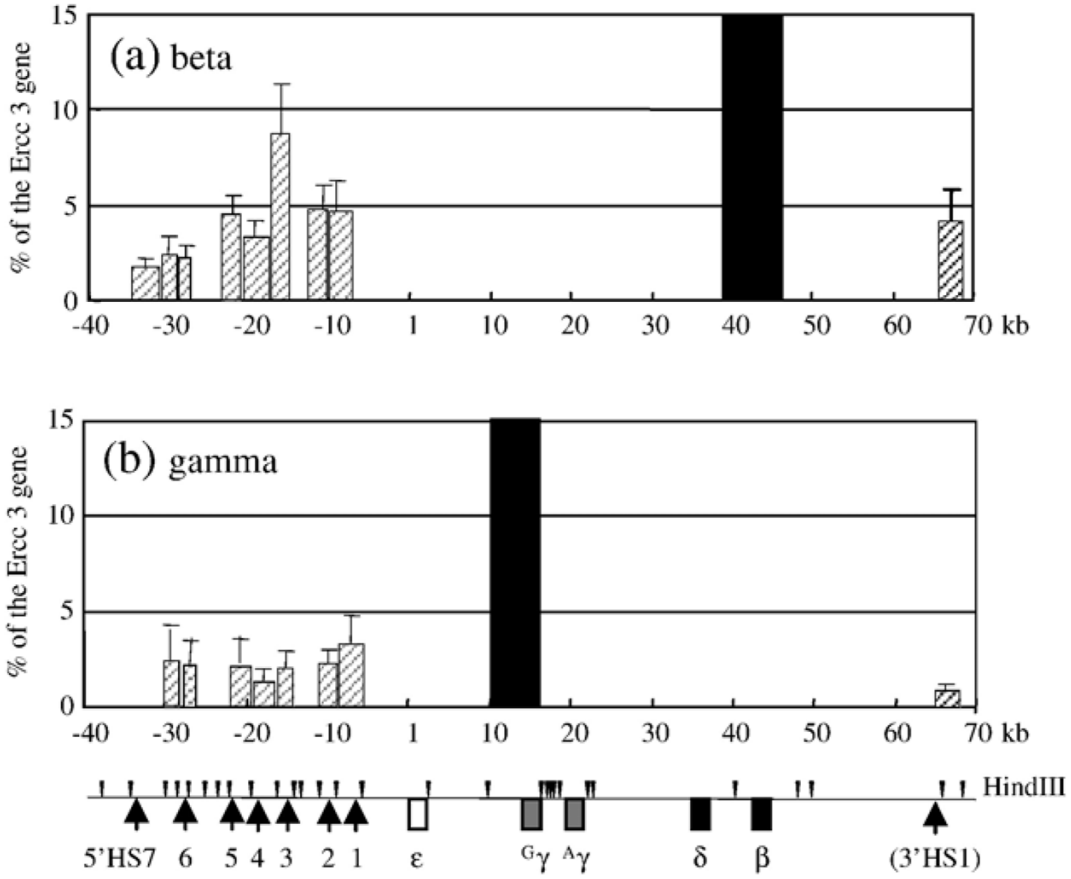

Figure 1.

The interaction of the distant 5′ and 3′ HS sites with the β or γ-globin gene in adult erythroblasts. The line drawn on the bottom shows the positions of the globin genes (in boxes), HS sites (in arrows), and HindIII sites (in small arrow heads). The 3′HS1 is not formed in transgenic mice and is shown in parenthesis. The width of the striped bars represents the size of the measured HindIII fragment and its height indicates the percentage of ligation frequency by setting the Ercc3 gene as 100%. The filled bars are the anchor fragments used in 3C assays. The *x*-axis shows the coordinates with the cap site of the ε-globin gene set as 1. (a) The β-globin gene is used as the anchor fragment. (b) The $^{G}\gamma$-globin gene is used as the anchor fragment.

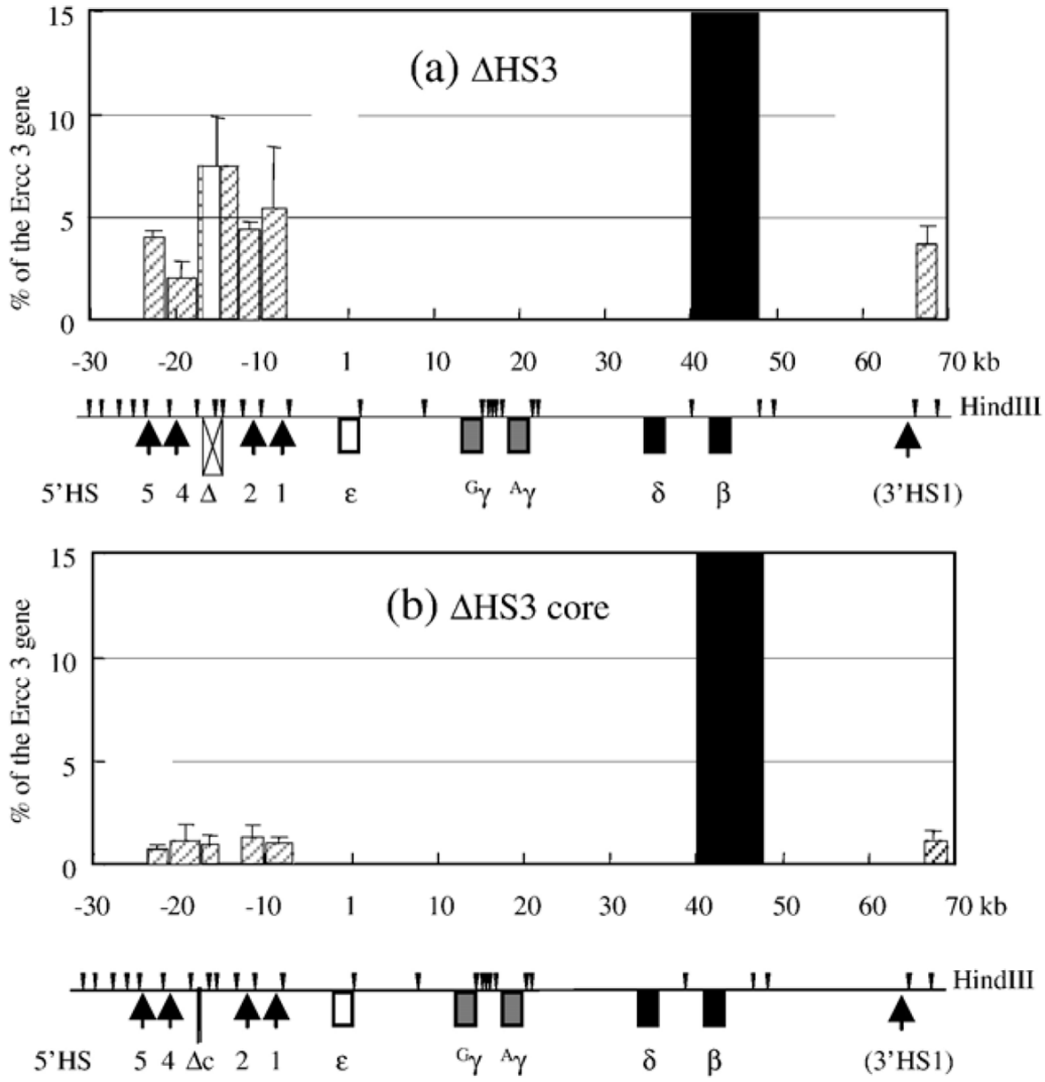

Figure 2.

The effects of the 2.3 kb HS3 deletion (a) and the 0.23 kb HS3 core deletion (b) on the interaction between the LCR and the β-globin gene. The 2.3 kb HS deletion removed two HindIII sites, and the deleted sequences are shown as the open bar. The resulting two franking fragments were fused, shown as the striped bars adjacent to the open bar in (a), and they represent the 3C result in this region. The 0.23 kb HS3 core deletion does not affect the primer location used in 3C assay. See the legend to Figure 1 for details.

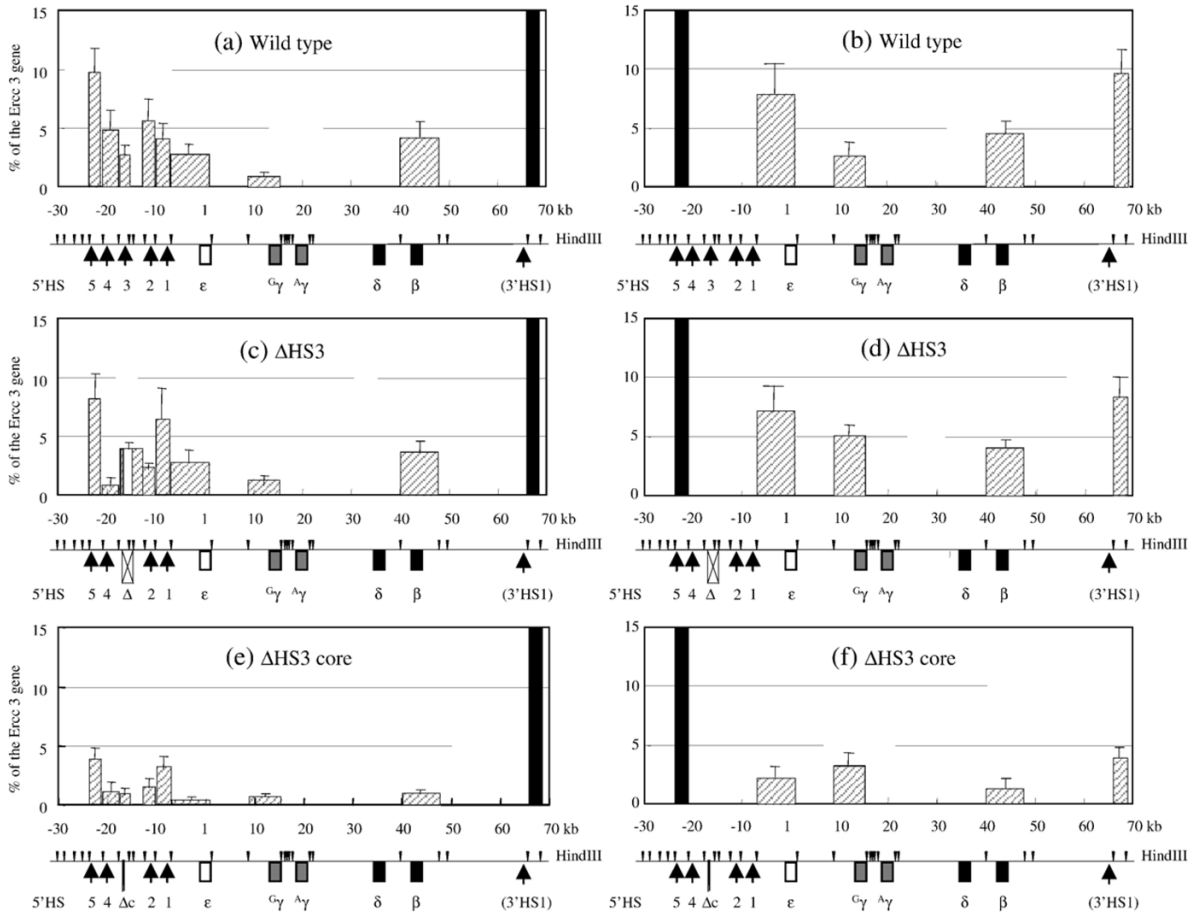

Figure 3.

The effects of the 2.3 kb HS3 deletion and the 0.23 kb HS3 core deletion on the interaction between 5′HS5 of the LCR and the 3′HS1 region. (a), (c) and (e) The results using 3′HS1 as the anchor fragment. (b), (d) and (f) The results using 5′HS5 as the anchor fragment. See the legends to Figures 1 and 2 for details.