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Differential requirement of a distal regulatory region for pre-initiation complex formation at globin gene promoters

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ABSTRACT

Although distal regulatory regions are frequent throughout the genome, the molecular mechanisms by which they act in a promoter-specific manner remain to be elucidated. The human β-globin locus constitutes an extremely well-established multigenic model to investigate this issue. In erythroid cells, the β-globin locus control region (LCR) exerts distal regulatory function by influencing local chromatin organization and inducing high-level expression of individual β-like globin genes. Moreover, in transgenic mice expressing the entire human β-globin locus, deletion of LCR-hypersensitive site 2 (HS2) can alter β-like globin gene expression. Here, we show that abnormal expression of human β-like globin genes in the absence of HS2 is associated with decreased efficacy of pre-initiation complex formation at the human ε- and γ-promoters, but not at the β-promoter. This promoter-specific phenomenon is associated with reduced long-range interactions between the HS2-deleted LCR and human γ-promoters. We also find that HS2 is dispensable for high-level human β-gene transcription, whereas deletion of this hypersensitive site can alter locus chromatin organization; therefore the functions exerted by HS2 in transcriptional enhancement and locus chromatin organization are distinct. Overall, our data delineate one mechanism whereby a distal regulatory region provides promoter-specific transcriptional enhancement.

INTRODUCTION

The precise regulation of multiple genes through the intermediacy of both proximal and distal *cis*-regulatory regions is prerequisite for normal development and cellular differentiation. Specific transcription factors (TFs) initially bind these regions and favor the recruitment of yet other TFs and co-factors. The combinatorial effect of these factors then modulates chromatin remodeling, covalent modification of histones and/or long-range chromatin interactions that facilitate either gene-activation or -repression (1–6).

The human β- (huβ-) globin locus provides a powerful model to investigate the roles of *cis*-regulatory regions and TFs. This locus is comprised of five developmentally regulated genes: embryonic ε-, fetal ^{G}γ- and ^{A}γ- as well as adult δ- and β-globin genes. Mutations affecting either expression of the huβ-globin gene, or composition of β-globin chains, can result in β-thalassemia or sickle-cell anemia. Treatments for these diseases aim primarily at the transcriptional reactivation of fetal globin genes in adult bone marrow, but are not always efficient (7). As such, the development of new molecular therapeutic strategies to stimulate huβ-globin locus transcription is considered an important challenge for the future.

Transgenic mice carrying the complete huβ-globin locus express the ε- and γ-genes in primitive erythroid cells (EryCs) derived from yolk sac. Around embryonic day 11 (e11), fetal liver EryC primarily express huγ-genes. Soon after, during γ-to-β-globin switching, γ-gene transcription progressively decreases which is concomitant with β-gene transcriptional activation (8). In general, specific TFs and co-factors recruited to the β-globin locus influence locus chromatin organization and/or promote long-range chromatin interactions and, as such, are

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critical for timely transcriptional activation/repression of β-like globin genes (9–13). For example, GATA-1 and erythroid Krüppel-like factor (EKLF) recruit the histone acetyltransferase CREB-binding protein (CBP), the histone deacetylase HDAC1 (14–18) and various chromatin remodeling complexes to the locus (18–20). EKLF is required for the recruitment of E-RC1 (erythroid-remodeling complex 1) to adult β-like globin gene promoters (21). The p45 subunit of NF-E2 (p45), another TF important for regulation of the huβ-globin locus, can also bind CBP (22), HDAC1, and the chromatin remodeling factor BRG1 (23). Finally, in addition to their roles in facilitating histone covalent modification, GATA-1 and p45 were shown to interact with specific pre-initiation complex (PIC) subunits (24,25).

The erythroid-specific β-globin LCR, composed of five DNaseI hypersensitive sites (HS) located 5′ of the locus, induces high-level globin gene expression by mediating long-range chromatin interactions that maintain close proximity between the LCR and active globin gene promoters (26–29). In humans (30) and transgenic mice (31,32), due to position effects, truncated LCRs are not able to prevent abnormal gene expression as efficiently as complete ones. For instance, in EryC derived from transgenic mice lacking HS2, huβ-like globin genes are abnormally regulated when the transgene is integrated within pericentromeric regions (31). In murine fetal and adult EryC, the abnormal pattern of huβ-gene expression is caused by position effect variegation (PEV) (31,33), characterized by stochastic repression of transcription in a subset of cells due to the spreading of heterochromatin from genomic sites adjacent to the transgene (34).

The LCR has been proposed to act as a holocomplex (35,36). Deletion of any among the five HS regions can destabilize this holocomplex, thereby preventing long-range chromatin interactions critical for high-level globin gene transcription (37,38). However, molecular dissection of the LCR strongly suggests that each HS exerts distinct functions. For instance, HS2 alone can enhance globin gene expression in EryC, although it does not protect transgenes from position effects (39). It has also been reported that globin gene expression can be influenced differentially depending upon the precise HS region deleted (31,36,37,40–43). Indeed, transgenic mice carrying an HS2 deletion were characterized by decreased huγ- and huβ-globin gene expression in yolk sac-, fetal liver- as well as bone marrow-derived EryC (36), although dissenting studies revealed no significant effect on expression of these genes in early fetal liver EryC (37). Finally, when the endogenous murine HS2 is deleted, embryonic genes are barely affected while the expression of adult genes is decreased by up to 30% (42,44). Therefore, the influence of HS2 over globin gene expression might be variable during ontogeny, and the role of HS2 in mediating transcriptional enhancement remains to be further clarified.

It has been proposed that HS2 is involved in PIC nucleation, since TATA-binding protein (TBP) and the general transcription factor IIB (TFIIB) are recruited to HS2 in both bone marrow hematopoietic progenitor cells and EryC (33,45). In addition, Johnson *et al.* (46) suggested that in EryC, phosphorylated RNA polymerase II (Pol II) is recruited to HS2 and then transferred to globin gene promoters. These data suggest that the LCR, and more specifically HS2, might enhance transcription by facilitating recruitment of PIC constituents that, in turn, contribute to PIC formation and stability at globin gene promoters. Moreover, HS2 was shown to be important for the recruitment of histone modifying complexes and chromatin remodeling activities (47,48), suggesting that in addition to its enhancer activity this hypersensitive site may be involved in locus chromatin organization. However, it is not clear if HS2 promotes PIC formation and chromatin reorganization activities at the same time, or if these events are coordinated separately during development.

Here, we have characterized the molecular mechanisms by which HS2 affects globin locus chromatin organization and globin gene expression in EryC isolated from yolk sac at e10.5 and from fetal liver at e12.5. Specifically, we investigated (i) recruitment of TFs, chromatin-modifying factors and PIC components, in addition to (ii) long-range chromatin interactions in transgenic mice containing the entire human β-globin locus deleted or not for the HS2 region. Our results demonstrate that HS2-enhancer activity can be exerted in a promoter-specific manner, and that the capacities of HS2 to induce transcriptional enhancement and locus chromatin organization are distinct.

MATERIALS AND METHODS

Transgenic mouse lines

Ln2 homozygous embryos (transgene copy number: 2) (8) were collected at e10.5 or e12.5. E10.5 embryos were washed twice in phosphate-buffered saline (PBS) and yolk sacs disrupted and punctured in order to collect blood cells; e12.5 fetal livers were isolated and homogenized in PBS. Δ2B homozygous mice (31) were crossed with CD1 females and EryC from heterozygous Δ2B (transgene copy number: 3) e10.5 yolk sacs or e12.5 fetal livers were isolated as described above. Finally, Δ2C homozygous mice (31) were bred together and homozygous $\Delta 2C^{+/+}$ (transgene copy number: 2) e10.5 yolk sacs or e12.5 fetal livers were isolated. All animal experiments were conducted in strict accordance with Canadian Council on Animal Care (CCAC) guidelines, and approved by the Maisonneuve-Rosemont Hospital animal care committee.

Chromatin immunoprecipitation (ChIP) and quantitative real-time PCR (qPCR) assays

ChIP assays were carried out as per manufacturer's instruction (Millipore). Briefly, cells were cross-linked in 1% formaldehyde for 10 min at 37°C. Cells were then incubated in lysis buffer containing 1 μg/ml aprotinin and 1 mM phenylmethanesulphonylfluoride (PMSF) and sonicated in order to obtain chromatin fragments of 400 bp on average. After centrifugation for 10 min at 13 000 r.p.m., chromatin was diluted 1:10 in the presence of protease inhibitors and pre-cleared for 30 min with salmon sperm DNA/Protein A agarose beads (Millipore).

Chromatin fragments were incubated with the appropriate antibodies overnight at 4°C. Beads were then washed with low-salt buffer, high-salt buffer, LiCl buffer and twice with TE buffer (Millipore). Chromatin fragments were eluted from beads in elution buffer (0.1 M $NaHCO_3$, 1% SDS). De-cross-linking was carried out for 4 h at 65°C. Following proteinase K treatment, DNA was phenol–chloroform extracted, precipitated with ethanol and resuspended in water. Antibodies used were against tetra-acetylated histone H4 (K3, K8, K12 and K16), di-acetylated histone H3 (K9 and K14), di-methylated histone H3 (K4) and HDAC1 (Millipore); TBP (SI-1), TFIIB (C-18), p45 (C-19), GATA-1 (N6), Pol II (N-20), CBP (A-22) and BRG1 (H-88) (Santa Cruz); unmodified histone H1 (B419 AE-4) and di-methylated histone H3 (K9) (Abcam); and polyclonal anti-EKLF (kindly provided by S. Philipsen) (49). All antibodies were raised in rabbit except H1 and GATA-1 which, respectively, were raised in mouse and in rat. Isotype matched immunoglobin G (IgG) (SantaCruz) were used as control. About 1/30th of immunoprecipitated and unbound (input) material was used as template for duplex semiquantitative hot PCR (AcH3, AcH4, meK4 and TBP ChIP) or qPCR (meK9, H1, TFIIB, Pol II, EKLF, GATA-1, p45, BRG1, CBP, HDAC1 and IgG ChIP) with one primer set specific for human β-globin regions (HS3, HS2, huε-, huγ- and huβ-globin gene promoters and coding regions) and another primer set specific for the internal control. For duplex quantitative PCR, reactions were performed in parallel under conditions of linear amplification and products were quantified by PhosporImager; *Zfp37* (neural-specific Zinc-finger protein 37) was used as internal control (3,50); *Thp* (Kidney-specific Tamm-Horsfall protein) (51) as negative control; and mouse HS2 (mHS2; e10.5 EryC) or *β major* (βmaj; e12.5 EryC), as positive controls. For qPCR, reactions were performed using SYBR Green (Invitrogen) with the iCycler iQ^{TM} (BioRad) system; *Thp* was used as internal control, amylase 2.1y [*amy*, (30)] as negative control and mHS2 or βmaj as positive controls. Since *Zfp37, Thp* and *amy* are repressed in EryC, for H1, meK9 and HDAC1 ChIP, *Thp* was used as positive control relative to *Gapdh* (active in EryC) and mHS2 or βmaj were used as negative controls. Quantification was carried out according to the $2^{-\Delta\Delta Ct}$ method. Primer sequences are available on request.

Quantitative RT-PCR (RT-qPCR)

Total RNA was isolated by Trizol (Invitrogen) and used for cDNA synthesis with oligo$(dT)_{12-18}$ or random primers and SuperScript Reverse Transcriptase III (Invitrogen). qPCR was carried out with QuantiTect probes specific for huβ- or huγ-globin cDNA (33). Intronic or LCR regions and mouse actin or GAPDH transcripts were detected by SYBR Green (Invitrogen). The following equation (52) was employed for quantification and the ratio corrected for transgene copy number:

$$\text{ratio} = \frac{(E_{\text{target}})^{\Delta CP_{\text{target}}(\text{control}-\text{sample})}}{(E_{\text{ref}})^{\Delta CP_{\text{ref}}(\text{control}-\text{sample})}}$$

E_{target}: target qPCR efficiency; E_{ref}: control qPCR efficiency; CP: crossing point; ΔCP_{target}: CP deviation of ln2 versus Δ2B lines target gene transcript; ΔCP_{ref}: CP deviation of ln2 versus Δ2B lines mouse control transcript. Primer sequences are available on request.

RNA–Fluorescent *in situ* hybridization (FISH)

RNA–FISH was performed as described in Wijgerde *et al.* (53) and van de Corput *et al.* (54). A detailed procedure is available as Supplementary Data (Figure S1).

DNaseI sensitivity assay

DNaseI sensitivity assay was carried out as previously described (50). About 300 000 nuclei were digested with up to 0.3 U of DNaseI (Roche, Indianapolis) for 30 min on ice and purified DNA was used as template for qPCR. Average molecular weight of DNaseI-treated samples was determined by Southern blotting.

Chromosome conformation capture (3C)

Chromosome conformation capture was carried out as described in Dekker *et al.* (55) and Bottardi *et al.* (56). A detailed procedure is available as Supplementary Data (Figure S1).

Hemoglobin staining

Intracellular staining was carried out on e12.5 fetal liver cells isolated from either ln2 or Δ2B mice. Ten to twenty million cells were fixed in 2% formaldehyde for 15 min at room temperature, and washed once with PBS and once with PBS/5% heat-inactivated fetal bovine serum (FBS) (PBS/FBS). For ChIP and DNaseI assays, cells were then permeabilized with 1 ml of PBS/FBS/0.5% saponin for 10 min at room temperature and stained with 20 μg of fluorescein isothiocyanate (FITC)-conjugated anti-HbA antibodies (clone 37-8, Santa Cruz) for 30 min at room temperature. Cells were washed in PBS/FBS/0.5% saponin and then again in PBS/FBS, and sorted by high-speed fluorescence-activated cell sorting (FACS) (FACS Vantage with DIVA option; Becton Dickinson, San Jose, CA, USA). To select for Δ2B EyC, which do not express the human hemoglobin β-chain (HbA^- cells), anti-HbA antibodies were coupled to rat anti-mouse Ter119 antibodies, followed by allophycocyanin (APC)-conjugated goat anti-rat IgG (Leinco Technologies) staining to isolate $Ter119^+HbA^-$ cells. Indeed, Ter119 antibody recognizes maturing erythroid cells from the proerythroblast stage onward (57). For variegation study, 1 million e12.5 fetal liver cells were permeabilized in 90% methanol for 10 min on ice. Cells were then stained with 1 μg of either FITC-conjugated anti-HbA antibody (as above) or FITC-conjugated anti-HbF antibody (clone 51-7, Santa Cruz). Cells were washed once in PBS/FBS and analyzed by FACScan (Becton Dickinson Immunocytometry System (BDIS), San Jose, CA, USA). These anti-HbA and anti-HbF do not cross-react with the murine globin chains (data not shown).

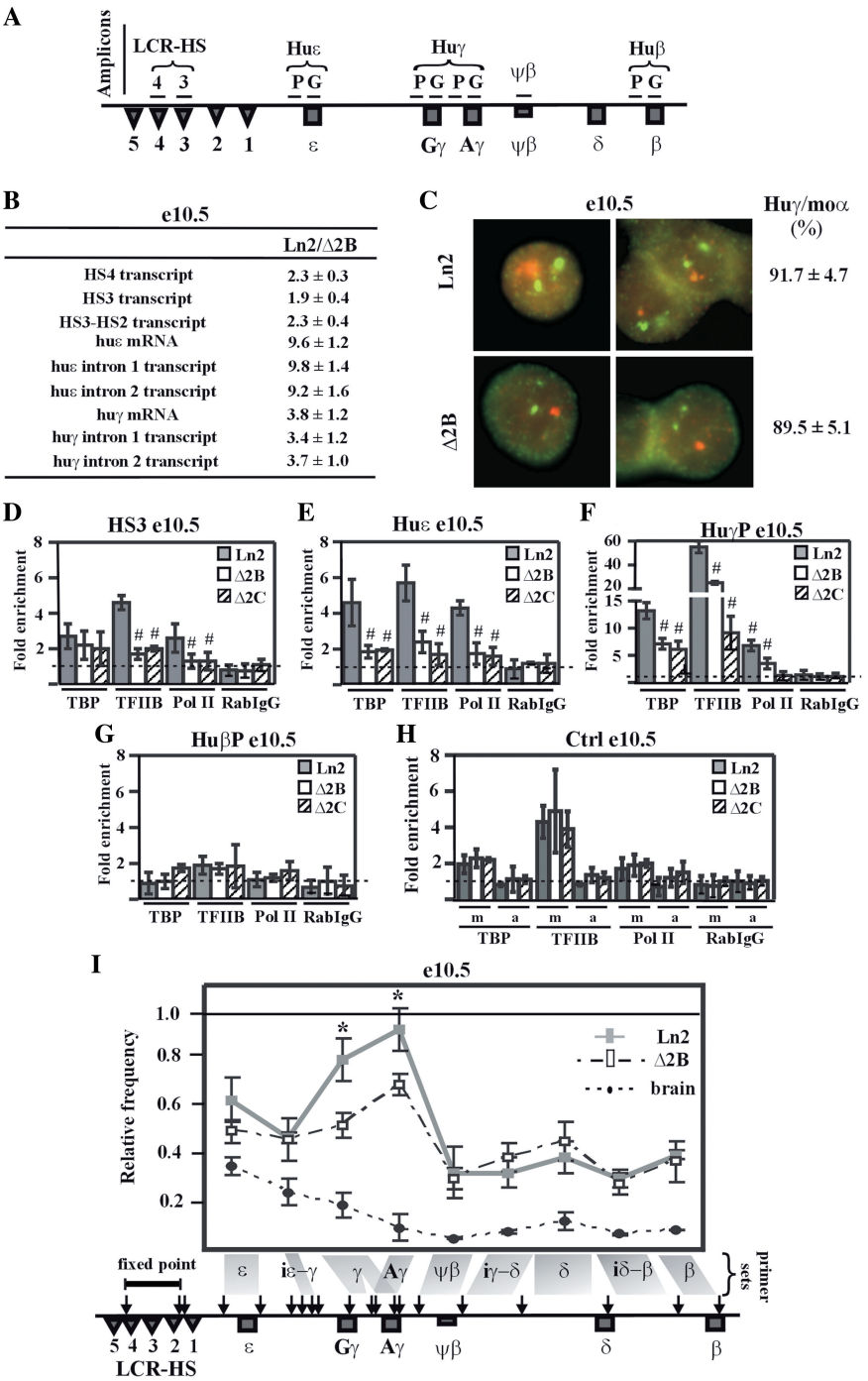

Figure 1. HS2 deletion affects PIC formation at huγ-promoters and long-range interactions among LCR and huγ-globin genes in e10.5 EryC. (**A**) Schematic representation of the human β-globin locus. Regions (amplicons) analyzed by ChIP are represented by horizontal lines. Due to high sequence similarity at huγ-promoters and at huγ-genes, the same primer sets amplify both the $hu^{A}\gamma$- and $hu^{G}\gamma$-promoters or genes; (**B**) RNA purified from ln2 and Δ2B e10.5 yolk sacs (e10.5) was retro-transcribed. Transcript quantification was made by qPCR, and relative levels of transcription (Ln2/Δ2B) were calculated according to Pfaffl (52) using mouse actin cDNA as internal control; HS4 or HS3 transcript: HS4 or HS3 primary transcript; HS3-HS2 transcript: primary transcript of the intergenic region between HS3 and HS2; huε mRNA: huε-globin mRNA transcript; huε intron 1 or 2 transcript: ε-globin intron 1 or 2 primary transcript; huγ mRNA: huγ-globin mRNA transcript; huγ intron 1 or 2 transcript: γ-globin intron 1 or 2 primary transcript (both $^{A}\gamma$ and $^{G}\gamma$ transcripts are amplified with the primer sets used); (**C**) Representative example of RNA–FISH on ln2 and Δ2B e10.5 yolk sacs (e10.5). Green signals: mouse α-globin primary transcript (moα; FITC detection); red signals: huγ-globin primary transcript (huγ; Texas Red detection; both $^{A}\gamma$ and $^{G}\gamma$ are recognized by the probes); (**D–H**) ChIP assays were carried out on e10.5 yolk sacs (e10.5; gray bars: ln2; white bars: Δ2B; dashed bars: Δ2C). Immunoprecipitated and input chromatin samples from TBP ChIP were subjected to duplex semiquantitative hot PCR and from TFIIB, Pol II and RabIgG (rabbit IgG) ChIP to qPCR. Fold enrichment (*y*-axis) of globin regions relative to the control and input samples are represented by bars, with corresponding standard deviations. A value of 1 (dashed line) indicates no enrichment. The positive control for TBP, TFIIB and Pol II ChIP is represented by m (mouse β-globin HS2; *mHS2/Thp*) and the negative control by a (*amy/Thp*); hash sign (#): $P \leq 0.05$ according to Student's *t*-test (ln2 versus Δ2B or ln2 versus Δ2C). The regions analyzed are specified on each

RESULTS

Globin gene expression in ln2 and Δ2B yolk sac EryC

To define the role of HS2 on globin gene expression during ontogeny, we investigated the effect of HS2 deletion on huε- and huγ-gene expression in e10.5 yolk sac and e12.5 fetal liver EryC derived from ln2 and Δ2B mice. Ln2 transgenic mice carry the 70-kb huβ-globin locus (Figure 1A), and express high-levels of huβ-like globin genes in a developmentally regulated manner (8). The Δ2B transgenic mice carries the same construct as ln2 except for a deletion of a 742-bp ApaLI–SnaBI fragment encompassing HS2, and they express huβ-like globin genes abnormally at all stages of development (31). Accordingly, quantitative RT-PCR (RT-qPCR) analysis indicates that in ln2 e10.5 EryC, huε- and huγ-globin messenger RNA (mRNA) levels are, respectively, ∼10-fold and ∼4-fold higher relative to Δ2B (Figure 1B). The pattern of huγ-gene expression was also investigated at the single-cell level by RNA–FISH using mouse α-globin transcript as internal control. We observed that huγ-genes are transcribed in all Δ2B e10.5 EryC because EryC, which can transcribe the endogenous murine α-globin gene, are also competent in huγ-gene transcription (Figure 1C). This indicates that in contrast to the PEV expression pattern that characterizes huβ-gene expression in Δ2B e13.5 fetal liver and bone-marrow-derived EryC (where only 25% of EryC express the huβ-gene) (31), abnormal huγ-gene expression in Δ2B e10.5 EryC is not attributable to PEV.

Toward understanding the origin of abnormal huβ-like globin gene expression in e10.5 EryC, we investigated huε- and huγ-primary transcript levels. In addition, since the LCR has been reported to influence transcriptional elongation of globin genes (58), we set out to determine whether the impaired huε- and huγ-gene expression in Δ2B e10.5 EryC could be related to abnormal transcriptional elongation. We observed that for both genes (i) primary transcript levels decrease in Δ2B cells but do not vary between introns 1 and 2 and (ii) the ratio of primary gene transcripts versus mRNA is similar in ln2 and Δ2B e10.5 EryC (Figure 1B). Taken together, these results indicate that decreased huε- and huγ-gene transcription in Δ2B e10.5 EryC is not a consequence of variegated expression, but rather is due to the absence of HS2, which alters expression levels without affecting transcriptional elongation.

PIC formation and long-range chromatin interactions at the huβ-like globin locus in ln2 and Δ2B yolk sac EryC

To elucidate the mechanism(s) influencing human globin gene expression in Δ2B e10.5 yolk sac EryC, we first studied PIC formation at huε- and huγ-promoters. Recruitment of the PIC subunits (59) TBP, TFIIB and Pol II was assessed by ChIP assay on ln2 and Δ2B e10.5 EryC. Since regulation of the mouse β-globin locus is normal in all transgenic mouse lines utilized, mouse LCR HS2 was used as positive control, and amylase as negative control, for ChIP assays (Figure 1H). As shown in Figure 1D–F, reduced detection of TBP, TFIIB and Pol II at HS3, huε-, and huγ-promoters suggests that PIC formation or stability is aberrant in the absence of HS2 in Δ2B e10.5 EryC.

Since it is proposed that long-range chromatin interactions are important for PIC formation/stability at promoters (46), we analyzed the interactions between the LCR and different regions across the β-globin locus using the chromosomal conformation capture (3C) assay (55). Nuclei were treated with formaldehyde, and the cross-linked genomic DNA digested and ligated. Genomic DNA fragments located in close proximity are more susceptible to ligation, and the frequency of ligation events can be detected by qPCR using appropriate primer sets (Figure 1I). Interestingly, we observed that HS2 deletion specifically affects long-range chromatin interactions between LCR and huγ-genes in Δ2B e10.5 EryC (Figure 1I), which is consistent with altered PIC formation at huγ-promoters and low-level huγ-gene transcription in these cells. Possibly due to their linear proximity, no significant variation in frequency of contact between LCR and the huε-region could be detected.

In order to further understand the basis for abnormal globin gene expression in Δ2B e10.5 EryC, we investigated EKLF, GATA-1 and p45 recruitment to the β-globin locus (Figure 2A–D). As shown in Figure 2C, only p45 recruitment to huγ-promoters is significantly affected in Δ2B cells. Among the co-factors interacting with p45 and capable of influencing locus organization (23), we noted a reduction and increase, respectively, of BRG1 and HDAC1 recruitment to huε- and huγ-promoters (Figure 2B and C). Thus, in addition to being important for PIC formation/stability, and for proper long-range chromatin interactions between the LCR and huγ-promoters in e10.5 EryC, the presence of HS2 appears to be required to ensure recruitment of p45 and BRG1 to huε- and huγ-promoters. In summary, our results indicate that HS2 is firmly involved in transcriptional enhancement of huε- and huγ-genes in e10.5 EryC.

PIC formation and long-range chromatin interactions at the huβ-globin locus in ln2 and Δ2B fetal liver EryC

Based on the above results, we investigated whether altered PIC formation and impaired long-range chromatin interactions might also take place in Δ2B e12.5 fetal liver EryC, where huγ- and huβ-globin genes (both active at

graph and the antibodies used for ChIP assays are indicated underneath each graph; (**I**) 3C assay performed on e10.5 yolk sacs (e10.5; gray line: ln2; dashed line: Δ2B). 3C ligation products were used as templates for qPCR analysis of the regions indicated on the *x*-axis. EcoRI digestion sites are shown by vertical arrows on the locus and fragments of interest are delimited by gray boxes. A primer set is formed by the combination of HS4-HS2 primer (fixed point) and one of the following primers: ε: huε-gene; iε-γ: intergenic region between huε- and huγ-gene; γ: $hu^{G}\gamma$- and $hu^{A}\gamma$-genes; $^{A}\gamma$: $hu^{A}\gamma$-gene; ψβ: ψβ gene; iγ-δ: intergenic region between $hu^{A}\gamma$- and huδ-gene; δ: huδ-gene; iδ-β: intergenic region between huδ- and huβ-gene; β: huβ-gene. Relative cross-linking frequency (*y*-axis) of the fixed point with globin fragments was defined using naked DNA as control and normalized to mouse actin. A relative frequency of one was attributed to the highest cross-linking frequency; asterisk (*): $P \leq 0.001$ according to Student's *t*-test (ln2 versus Δ2B); e12.5 brain cells (dotted line), in which human β-like globin genes are not expressed were used as control.

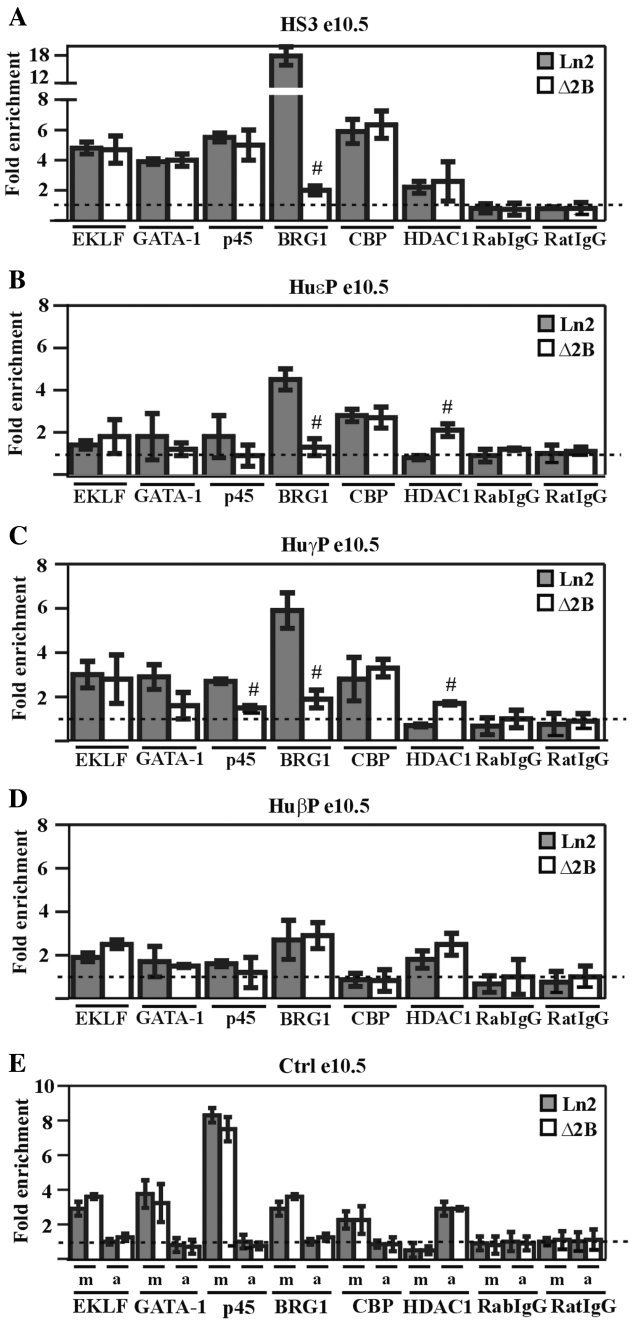

Figure 2. Transcription factor and co-factor recruitment at the huβ-globin locus in ln2 and Δ2B e10.5 EryC. (**A–E**) ChIP assays were carried out on e10.5 yolk sacs (e10.5; gray bars: ln2; white bars: Δ2B); immunoprecipitated and input chromatin samples were subject to qPCR. Fold enrichments were calculated as described in Figure 1 and are indicated on the *y*-axis; the positive control for EKLF, GATA-1, p45, BRG1 and CBP ChIP is represented by m (*mHS2*/*Thp*) and for HDAC1 ChIP, by a (*amy*/*Gapdh*). The negative control for EKLF, GATA-1, p45, BRG1 and CBP ChIP is represented by a (*amy*/*Thp*) and for HDAC1 ChIP, by m (*mHS2*/*Thp*). Hash sign (#): $P \leq 0.05$ according to Student's *t*-test (ln2 versus Δ2B). The regions analyzed are specified on each graph and the antibodies used for ChIP assays are indicated underneath each graph.

this developmental stage (8)) are characterized by a PEV pattern of expression (31). We observed that TBP recruitment to HS3, huγ- and huβ-promoters is not significantly affected in Δ2B e12.5 EryC, whereas TFIIB recruitment and Pol II loading are clearly less efficient in Δ2B than in ln2 cells (Figure 3C–E). TBP binding was confirmed in another PEV line, namely Δ2C (31), in which the same globin locus as Δ2B is integrated in a restrictive chromosomal environment that allows expression of the huβ-globin gene in only 4% of Δ2C EryC (Figure 3C–E and Figure S3C). Recruitment of TBP to a TATA box embedded in restrictive chromatin has been previously documented (60). However, due to reduced TFIIB and Pol II recruitment to globin gene promoters, we believe that TBP binding is not sufficient to promote PIC formation in Δ2B e12.5 EryC.

As shown in Figure 3G, the 3C analysis reveals that even though the LCR is frequently located in close proximity to huγ- and huβ-promoter regions in Δ2B e12.5 EryC, such interactions are significantly reduced compared to ln2 cells. Since it has been reported that TFs are important for PIC formation and long-range chromatin interactions, we investigated whether any TFs involved in chromatin organization and/or long-range interactions could be abnormally recruited to the β-globin locus. We observed that EKLF and GATA-1 recruitment to huγ- and huβ-promoters is reduced in Δ2B e12.5 EryC compared with the situation for ln2 cells (Figure 4B and C). This correlates with the PEV pattern of huγ- and huβ-gene expression in Δ2B e12.5 EryC (Figure 3A and B). p45 can assist GATA-1 during globin gene activation (24), and it is therefore interesting that p45 recruitment is also reduced at both huγ- and huβ-promoters (Figure 4B and C). We also observed reduced CBP and increased HDAC1 recruitment, especially at HS3 and the huβ-promoter (Figure 4A and C). However, since EKLF, GATA-1, and p45 are equally recruited to HS3 in e12.5 EryC derived from both lines, it is likely that CBP and HDAC1 recruitment to HS3 is facilitated by TFs other than those immediately aforementioned, and that the binding of these yet-to-be identified TFs at HS3 is reduced in Δ2B e12.5 fetal liver EryC. In summary, in Δ2B fetal liver EryC, the PEV expression pattern is primarily associated with pronounced alteration of TFIIB, Pol II, as well as EKLF, GATA-1 and p45 binding to huγ- and huβ-promoters.

Chromatin organization at the huβ-globin locus in yolk sac and fetal liver EryC

The results presented so far indicate that in Δ2B e10.5 EryC, reduced huε- and huγ-globin gene expression is not the consequence of PEV; however, later during development huγ- and huβ-gene expression is subject to PEV in Δ2B EryC [see above and (31,33)]. As such, it was interesting to investigate whether or not chromatin organization at the Δ2B locus varies in e10.5 versus e12.5 EryC. We therefore carried out ChIP assays using antibodies directed against di-acetylated histone H3 (AcH3), tetra-acetylated histone H4 (AcH4) or di-methylated histone H3 (meK4), all of which are normally associated with transcriptionally prone chromatin (61). The active mouse LCR HS2 (m), *β major* promoter (βmaj) or the inactive *Thp* gene (T) or amylase promoter (a) were used as controls (Figures 5F and 6E).

A

	e12.5 Ln2/Δ2B
HS4 transcript	1.9 ± 0.3
HS3 transcript	2.8 ± 1.1
HS3-HS2 transcript	1.7 ± 0.2
huγ mRNA	8.0 ± 1.3
huβ mRNA	5.6 ± 0.9

B

	e12.5	
	Ln2	Δ2B
% HbF$^+$	29.1 ± 2.0	7.2 ± 1.8
% HbA$^+$	83.6 ± 4.1	38.5 ± 3.0

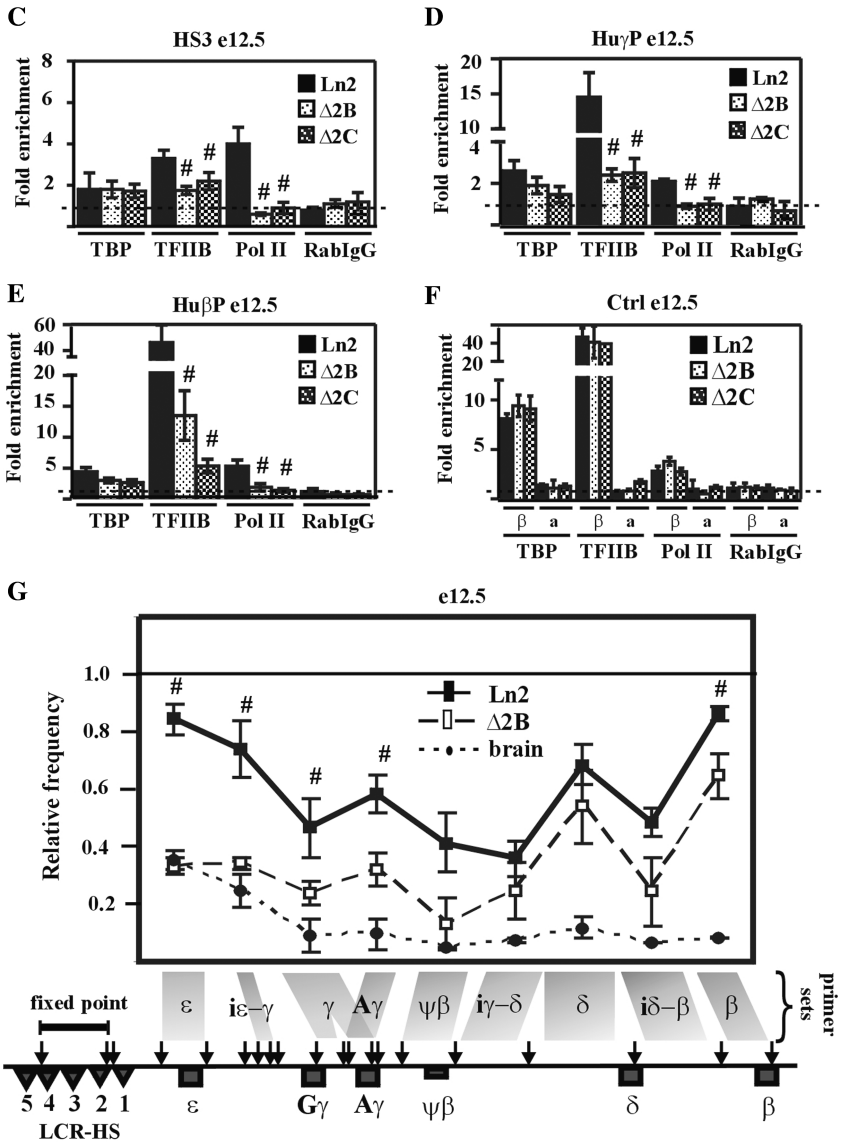

Figure 3. HS2 deletion affects PIC formation at huβ-like globin gene promoters and long-range chromatin interactions between LCR and huβ-like globin genes in e12.5 EryC. (**A**) RNA purified from ln2 and Δ2B e12.5 fetal liver EryC (e12.5) was retro-transcribed. qPCR was performed and analyzed as in Figure 1; (**B**) e12.5 fetal liver EryC were stained with anti-HbF or anti-HbA antibodies, as detailed in 'Materials and Methods' section; (**C–F**) ChIP assays were carried out on e12.5 fetal liver cells (e12.5; black bars: ln2; dotted bars: Δ2B; checked bars: Δ2C). Immunoprecipitated and input chromatin samples from TBP ChIP were subjected to duplex semiquantitative hot PCR and from TFIIB, Pol II and RabIgG (rabbit IgG) ChIP to qPCR. Immunoprecipitated and input chromatin samples were subject to qPCR. Fold enrichments were calculated as described in Figure 1 and are indicated on the *y*-axis. β (*βmaj*) control replaces mHS2 control for analysis in fetal liver EryC. Hash sign (#): $P \leq 0.05$ according to Student's *t*-test (ln2 versus Δ2B or ln2 versus Δ2C). The regions analyzed are specified on each graph and the antibodies used for ChIP assays are indicated underneath each graph; (**G**) 3C assay performed on e12.5 fetal liver cells (e12.5; black line: ln2; dashed line: Δ2B). 3C ligation products were used as templates for qPCR analysis of regions indicated on the *x*-axis. Relative cross-linking frequency (*y*-axis) of the fixed-point fragment (HS4-HS2) with huβ-globin fragments was defined as in Figure 1. Hash sign (#): $P \leq 0.05$ according to Student's *t*-test (ln2 versus Δ2B).

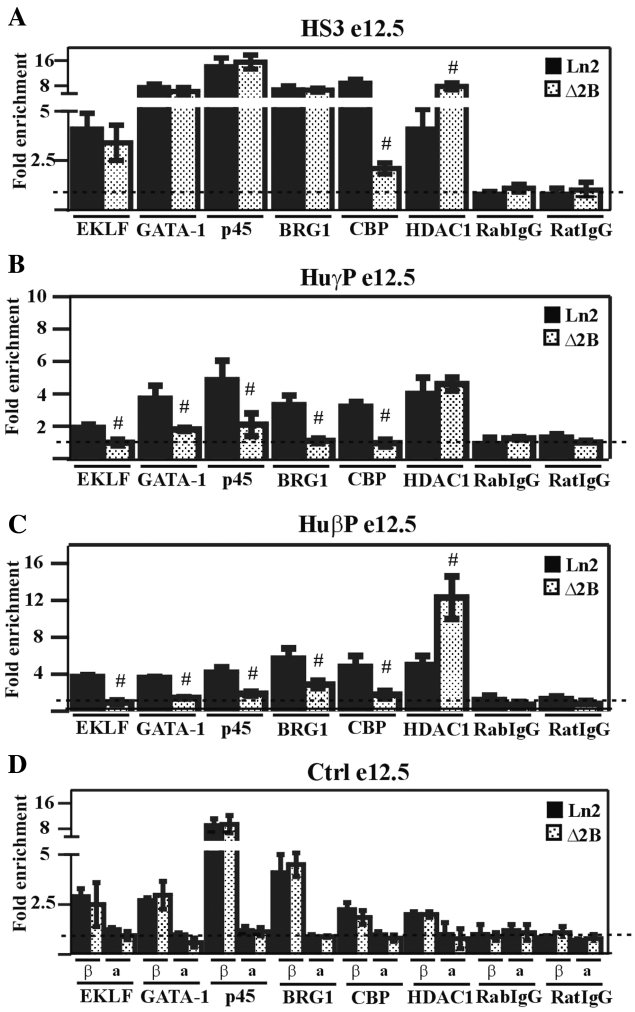

Figure 4. Transcription factor and co-factor recruitment at the huβ-globin locus in ln2 and Δ2B e12.5 EryC. (**A–D**) ChIP assays were carried out on e12.5 fetal liver EryC (e12.5; black bars: ln2; dotted bars: Δ2B. Immunoprecipitated and input chromatin samples were subject to qPCR. Fold enrichments were calculated as described in Figure 1 and are indicated on the *y*-axis. β (*βmaj*) control replaces mHS2 control for analysis in fetal liver cells. Hash sign (#): $P \leq 0.05$ according to Student's *t*-test (ln2 versus Δ2B). The regions analyzed are specified on each graph and the antibodies used for ChIP assays are indicated underneath each graph.

Figure 5A–C show that in ln2 e10.5 yolk sac EryC HS4, HS3, and the huε- and huγ-promoters and genes display, in general, high-levels of AcH3, AcH4 and meK4. Surprisingly, histone AcH4, known to be associated with active chromatin organization independent of gene transcriptional status (62), is very similar in ln2 and Δ2B loci. Moreover, AcH3 and meK4 levels at the inactive huβ-promoter and gene, as well as at the ψβ intergenic region (Figure 5D and E) are similar in ln2 and Δ2B and only local variations are detected at the LCR, and at the huε- and huγ-promoters and genes (Figure 5A–C). In both lines, histone H3 K9 methylation (meK9) or linker histone H1, both of which are associated with transcriptionally restricted chromatin (63,64), are not detected across the locus (Figure 5A–E). Finally, chromatin accessibility as assessed by DNaseI sensitivity assay is comparable in ln2 and Δ2B e10.5 EryC (Figure S2 A–E). In summary, since chromatin organization is very similar in Δ2B and ln2 cells and manifests no feature of transcriptionally restricted chromatin, it is unlikely that low-level huε- and huγ-gene expression in Δ2B e10.5 EryC is related to PEV. Rather, the variations observed at the huε- and huγ-regions in Δ2B versus ln2 e10.5 EryC are best explained by altered transcription levels of huε- and huγ-genes (Figure 1B), rather than by restrictive chromatin organization of the β-globin locus *per se*.

Chromatin organization was next studied in e12.5 fetal liver EryC. As expected, ChIP assays revealed that throughout the locus AcH3, AcH4 and meK4 are significantly underrepresented in Δ2B versus ln2 e12.5 EryC, whereas histone H1 recruitment to HS3 and to the huβ-promoter is 2- to 3-fold higher in Δ2B compared with ln2 cells (Figure 6A and D). Notably, the level of meK9 at the huβ-promoter is 3-fold lower in Δ2B versus ln2 e12.5 EryC (Figure 6C). This could be explained by the fact that, although frequently observed in repressive chromatin, meK9 has also been associated with βmaj active transcription (65) and other transcriptionally active genes (66,67). In accord with our ChIP results, DNaseI sensitivity assays carried out on e12.5 fetal livers revealed that across the locus, chromatin is less accessible in Δ2B than ln2 EryC (Figure S2 F–I). Thus, unlike what is observed at e10.5, in Δ2B e12.5 EryC, several regions of the β-globin locus are characterized by decreased histone acetylation levels and H1 enrichment, which together are reminiscent of a restrictive chromatin organization typical of PEV. Overall, these results support a role for HS2 in locus chromatin organization in e12.5 fetal liver EryC.

Characterization of a Δ2B EryC subpopulation expressing the huβ-globin gene

The results shown thus far provide no information as to whether reduced gene expression, impaired PIC formation, and abnormal chromatin organization at huβ-like globin promoters in Δ2B e12.5 EryC might be a consequence of (i) the absence of chromatin remodeling and/or histone-modifying activities (due to HS2 deletion), or (ii) altered HS2-mediated enhancer activity as observed in Δ2B e10.5 EryC. To address this, we compared various chromatin-related endpoints in ln2 and Δ2B e12.5 EryC subpopulations that actively transcribe the huβ-gene. These cells were isolated using an anti-HbA (adult hemoglobin) antibody specific for the human hemoglobin β-chain (HbA^+). Huβ-like globin gene expression (by RT-qPCR), PIC formation (by Pol II and TFIIB ChIP), and chromatin organization (by AcH3 and AcH4 ChIP and DNaseI sensitivity assay) were compared in HbA^+ cells. As shown in Figure 7A, no significant variation of huβ-gene expression was detected in ln2 versus Δ2B HbA^+ cells. Accordingly, we observed that chromatin organization (Figure 7B and C; Figure S2 J–L) and PIC formation (Figure 7D and E) at the huβ-promoter are similar in ln2 versus Δ2B HbA^+ cells, while, as expected, PIC formation and histone acetylation levels are remarkably decreased in Δ2B $Ter119^+$ HbA^- cells (*i.e.*, EryC not

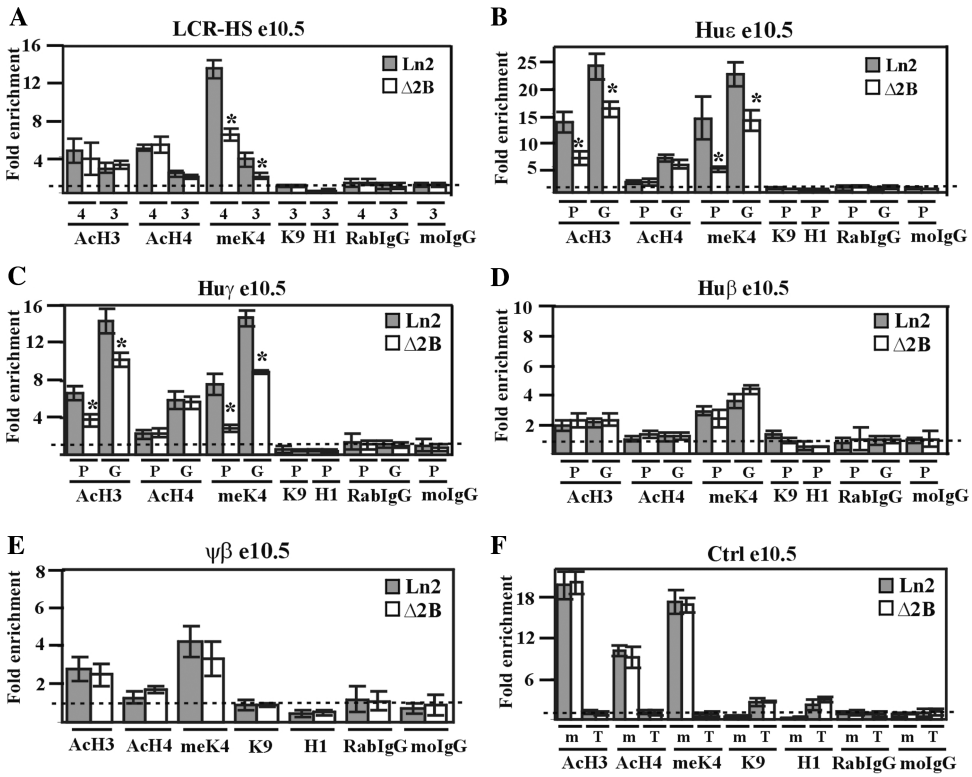

Figure 5. Effect of HS2 deletion on huβ-globin locus chromatin organization in e10.5 EryC. (**A–F**) ChIP assays were carried out on e10.5 yolk sacs (e10.5; gray bars: ln2; white bars: Δ2B). Immunoprecipitated and input chromatin samples from AcH3, AcH4 and meK4 ChIP were subjected to duplex quantitative PCR and from meK9, H1, RabIgG and moIgG ChIP were subjected to qPCR. Fold enrichment (*y*-axis) of globin regions relative to the control and input samples were calculated as described in Figure 1. The negative control for AcH3, AcH4 and meK4 ChIP is represented by T (*Thp*/*Zfp*) and for H1 and meK9 ChIP, by m (*mHS2*/*Thp*). The positive control for AcH3, AcH4 and meK4 ChIP is represented by m (*mHS2*/*Zfp*) and for H1 and meK9 ChIP, by T (*Thp*/*Gapdh*); asterisks (*): $P \leq 0.001$ according to Student's *t*-test (ln2 versus Δ2B). The regions analyzed are specified on each graph and the antibodies used for ChIP assays are indicated underneath each graph; 4 (HS4), 3 (HS3), P (promoter), G (gene); AcH3: di-acetylated histone H3; AcH4: tetra-acetylated histone H4; meK4: di-methylated lysine 4 histone H3; H1: Histone H1; meK9: di-methylated lysine 9 and lysine 27 histone H3.

transcribing the huβ-globin gene; Figure 7B–E). The above strongly suggests that the absence of HS2 enhancer activity does not affect the efficacy of PIC formation or chromatin organization at the huβ-promoter and, furthermore, that HS2 is not required for huβ-gene high-level transcription in e12.5 EryC. Surprisingly, huγ-gene transcription is reduced in Δ2B HbA^+ cells (Figure 7A). Consistent with this latter observation, chromatin organization at huγ-genes is impaired in these cells (Figure 7B and C; Figure S2 K) and TFIIB and Pol II are recruited less efficiently at huγ-promoters (Figure 7D and E). Thus, as for e10.5 EryC, HS2 deletion in e12.5 EryC precludes efficient PIC formation at huγ-promoters, but has no significant effect at the huβ-promoter. Since the transcription level of the huβ-gene is not affected in Δ2B HbA^+ EryC, even though huβ-gene expression is characterized by PEV in Δ2B e12.5 EryC, we conclude that the PEV expression pattern of the huβ-gene is not related to absence of HS2-mediated enhancer activity. Instead, impaired recruitment of histone-modifying or chromatin-remodeling activities to the locus due to HS2 deletion likely facilitates invasion of the locus by restrictive chromatin organization originating at the genomic integration site of the transgene.

DISCUSSION

The comparative analysis of huβ-like globin gene expression in e10.5 and e12.5 EryC undertaken here reveals that in absence of HS2, the huε- and huγ-promoters are characterized by impaired PIC formation as well as abnormal recruitment/stability of specific TFs and co-factors. However, at the same time, we show that HS2 is not required for huβ-gene transcriptional enhancement. Thus, the contribution of HS2 to PIC formation and promoter organization is required for high-level transcription of some but not all globin genes. Finally, we demonstrate that in Δ2B e12.5 EryC, HS2 deletion does not affect transcription levels of the huβ-gene but does facilitate the induction of abnormal chromatin organization over the locus. The above, taken together, indicates that HS2 functions separately in transcriptional enhancement and locus chromatin organization.

Influence of HS2 deletion on huε- and huγ-gene transcriptional enhancement

Here, we showed that low-level globin gene transcription in Δ2B e10.5 EryC is a consequence of impaired LCR enhancer activity due to HS2 deletion, and not of

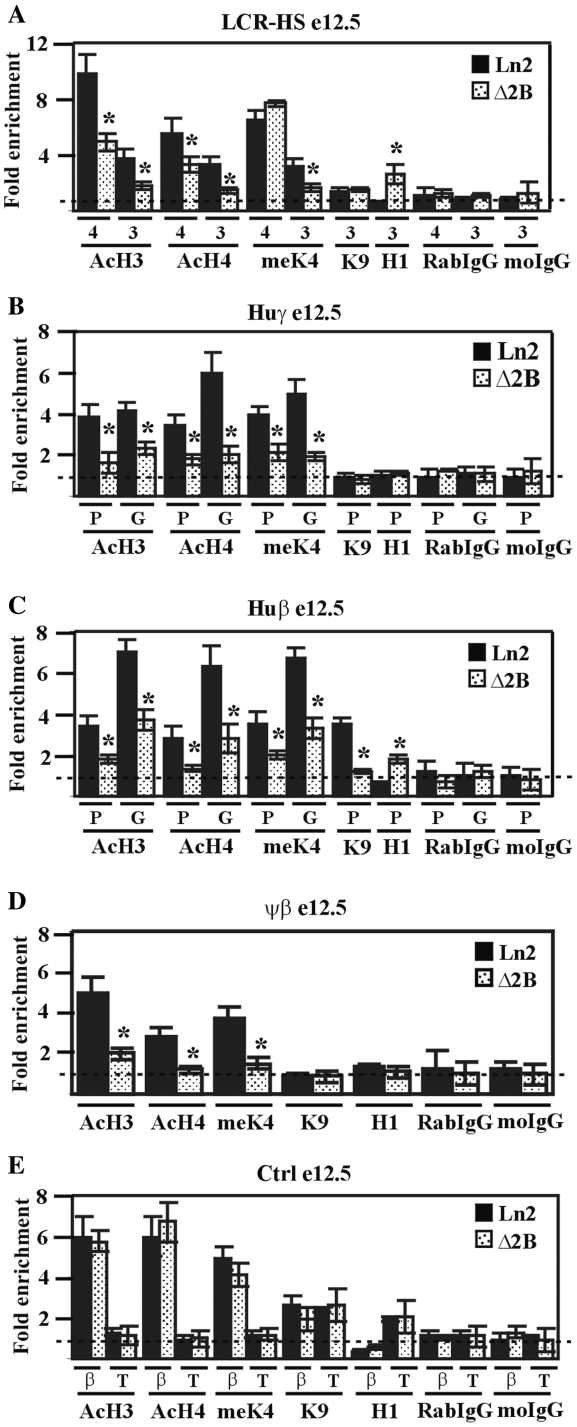

Figure 6. Effect of HS2 deletion on huβ-globin locus chromatin organization in e12.5 EryC. (**A–E**) ChIP assays were carried out on e12.5 fetal liver EryC (e12.5; black bars: ln2; dotted bars: Δ2B). Immunoprecipitated and input chromatin samples from AcH3, AcH4 and meK4 ChIP were subject to duplex quantitative PCR and from meK9, H1, RabIgG and moIgG ChIP were subjected to qPCR. Fold enrichments were calculated as described in Figure 1 and are indicated on the *y*-axis. β (*βmaj*) control replaces mHS2 control for analysis in fetal liver cells. The regions analyzed are specified on each graph and the antibodies used for ChIP assays are indicated underneath each graph; 4 (HS4), 3 (HS3), P (promoter), G (gene); AcH3: di-acetylated histone H3; AcH4: tetra-acetylated histone H4; meK4: di-methylated lysine 4 histone H3; H1: Histone H1; meK9: di-methylated lysine 9 and lysine 27 histone H3. Asterisk (*): $P \leq 0.001$ according to Student's *t*-test (ln2 versus Δ2B).

disruption of the active locus-wide chromatin organization. Indeed, in e10.5 EryC, chromatin at the huβ-globin locus is accessible and devoid of heterochromatin marks. The fact that human globin genes manifest a PEV expression pattern in e12.5 Δ2B fetal liver EryC but not in e10.5 yolk sac EryC is most likely related to developmental-stage-specific spreading of heterochromatin from the β-globin transgene integration site within the mouse genome.

We also showed that in Δ2B e10.5 EryC, variations in histone covalent modifications at huγ-promoters are associated with impaired LCR-huγ-promoter interactions. Such variations might be a consequence of abnormal transcription levels in Δ2B cells, since AcH3 [specifically at the β-globin locus (68)], as well as H3K4 di- and tri-methylation [at various genetic loci (69)], have been associated with high-level gene transcription. Alternatively, variations in histone covalent modifications might be linked to reduced recruitment of enzymatic activities by TFs such as p45 (2), which we showed occurs less efficiently at huγ-promoters in Δ2B compared with ln2 cells. In fact, the correlation between these latter variations, abnormal PIC formation, altered LCR/huγ-gene long-range chromatin interactions, and low-level huε- and huγ-gene transcription, might not be fortuitous. For instance, reduced p45 and BRG1 recruitment to huε- and huγ-promoters could be responsible for low huε- and huγ-gene expression levels in Δ2B e10.5 EryC since both p45 and BRG1 are required for efficient Pol II occupancy at globin promoters (19,24,70,71). Moreover, recently, BRG1 has been shown to affect long-range interactions at the β-globin locus (13). It should be noted that low AcH3 levels at huγ-regions are not related to CBP recruitment (Figures 5C and 2C), suggesting that effects on the recruitment/stability of other acetyltransferases binding the β-globin locus, e.g. p300 and PCAF (14,72) might be implicated.

Distal regulatory regions can enhance transcription by various means including modulation of both PIC formation and the efficacy of transcriptional elongation. In Δ2B e10.5 EryC, we observed a relatively low frequency of LCR/huγ-gene long-range interactions, decreased recruitment of PIC components to the LCR as well as altered PIC formation at huε- and huγ-promoters. Additionally, we did not detect significant variation in TF recruitment at HS3, nor in DNaseI sensitivity at HS3, in accord with what has been reported for the mouse β-globin locus (73). However, we did observe that primary transcription of HS4, HS3, and inter-HS3-HS2 regions is relatively reduced in Δ2B EryC (Figures 1B and 3A). Likewise, Pol II and TFIIB loading at HS3 (45) is decreased in these cells (Figures 1D and 3C). This indicates that HS2 is required for proper LCR primary transcription as well as for TFIIB and Pol II loading at the LCR. The above data, taken together, suggest that HS2 influences other regions of the LCR and, most likely, acts in synergy with other LCR hypersensitive sites. As previously proposed (45,74), it is possible that low-level LCR intergenic transcription influences the recruitment of other TFs to HS3 in Δ2B EryC. This is supported by the abnormal recruitment of BRG1 to HS3 in Δ2B e10.5 EryC (Figure 2A). Additionally, as shown for the human

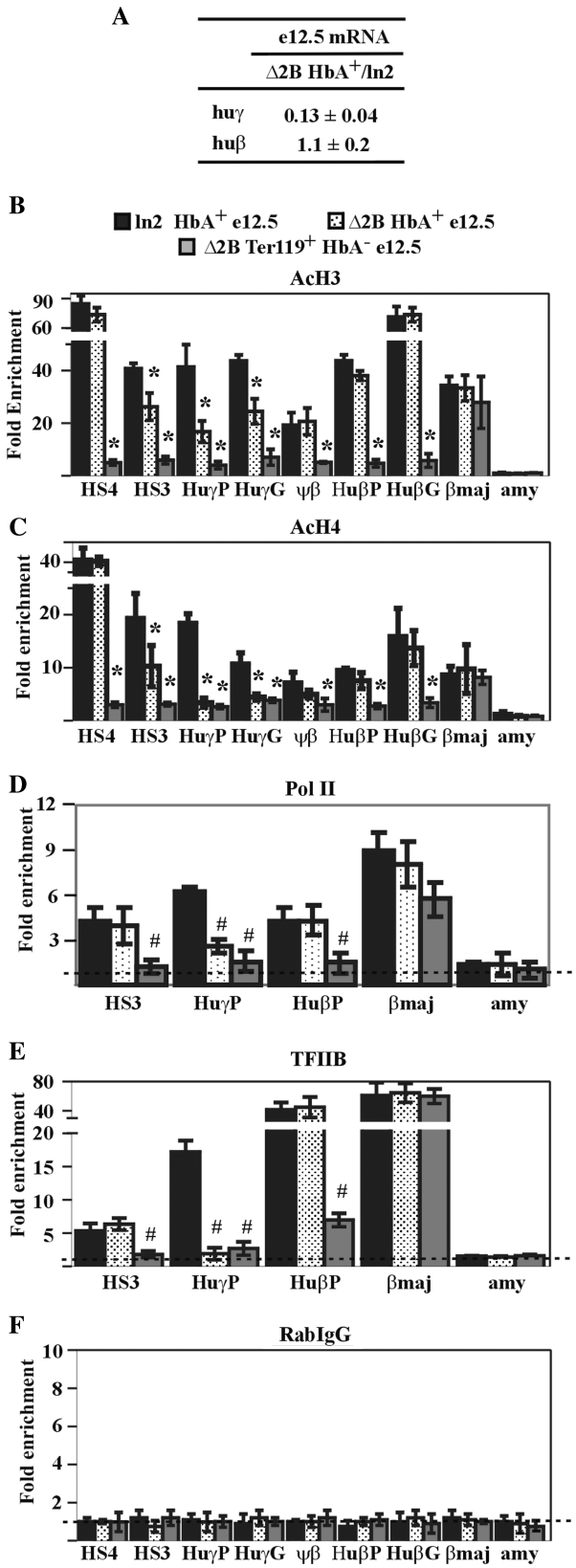

Figure 7. HS2 deletion specifically affects huγ-globin gene expression and chromatin organization in HbA^+ e12.5 EryC. Ln2 and Δ2B e12.5 fetal liver cells were sorted according to huβ-globin expression with an antibody recognizing the human β-globin chain of adult hemoglobin (HbA^+). (**A**) Equal amounts of RNA purified from Δ2B and ln2 HbA^+ cells were retro-transcribed. qPCR was performed and analyzed such as in Figure 1; huγ: huγ-globin mRNA; huβ:huβ-globin mRNA; (**B–F**) ChIP assays were carried out on ln2 and Δ2B HbA^+ e12.5 fetal liver cells (black bars: ln2; dotted bars: Δ2B) and on Δ2B $Ter119^+$ HbA^- e12.5 fetal liver cells (gray bars). Immunoprecipitated and input chromatin samples were subject to qPCR. Fold enrichments were calculated as described in Figure 1 and are indicated on the *y*-axis. Hash sign (#): $P \leq 0.05$ according to Student's *t*-test (ln2 HbA^+ versus Δ2B HbA^+ or ln2 HbA^+ versus Δ2B $Ter119^+$ HbA^-). The regions analyzed are specified on each graph and the antibodies used for ChIP assays are indicated underneath each graph.

growth hormone LCR (75), primary transcription of the β-globin LCR could potentially control its ability to participate in long-range chromatin interactions and gene activation.

Our data demonstrating that *trans*-acting factors are recruited less efficiently to huβ-like globin gene promoters in Δ2B e10.5 EryC, i.e. where the locus is not in restrictive chromatin organization, strongly suggests that the HS2-deleted LCR also affects TF and co-factor recruitment or stability at promoters. Since GATA-1 and p45 can be recruited to globin gene promoters in the absence of the LCR (11,76), and are reported to interact with some PIC subunits (24,25), it is likely that the efficacy of PIC formation (which is affected by HS2 deletion in Δ2B e10.5 EryC) influences the stability at least of p45 at huε- and huγ- promoters. Thus, we propose that the main function of HS2 in transcriptional enhancement is to promote PIC formation at huε- (77) and huγ-promoters. This is consistent with previous observations at the mouse and human β-globin loci, which suggest that LCR-bound Pol II can be transferred to globin gene promoters, and that HS2 is the major LCR site for recruitment of phosphorylated Pol II (46,71,78).

HS2 affects human β-like globin genes differentially and is involved in locus chromatin organization

The specific roles of the various LCR HS regions have been a matter of debate (31,36,37,41,43,79). In particular, it is not clear if HS2 promotes PIC formation and chromatin organization activities at the same time, or if these activities are coordinated separately during development. It was reported that in the context of a minimal construct where HS2 is linked to the β-globin gene, this hypersensitive region constitutes a poor modulator of chromatin organization but nonetheless retains significant transcriptional enhancer activity (39). Indeed, chromatin-modifying and -remodeling activities are recruited to HS2 (3,47,48) and influence chromatin organization across the β-globin locus (47,48). Additionally, HS2 deletion favors abnormal chromatin organization when the transgene is integrated within chromosomal regions marked by heterochromatin (31,32). Thus, a PEV pattern of expression in Δ2B e12.5 EryC could be facilitated by the absence of specific chromatin-modifying or -remodeling activities normally recruited to HS2 (3,47,48) and/or by reduced efficiency of PIC formation/stability at human β-like globin gene promoters. However, characterization of the Δ2B e12.5 EryC subpopulation expressing the huβ-globin gene (Δ2B HbA^+ EryC) indicates that the absence

of HS2 affects PIC formation at huγ-promoters, but not at the huβ-promoter. Since the huβ-gene has a characteristic PEV expression pattern in Δ2B e12.5 fetal liver EryC, but is nonetheless equally transcribed in ln2 and Δ2B HbA^+ EryC, it appears that alteration of HS2 enhancer activity is not required for PEV. This also argues in favor of the notion that HS2 enhancer- and chromatin-activation functions exhibit dichotomy with respect to one another (80,81).

HS3 is known to be important for Pol II recruitment to the huβ-promoter as well as for long-range chromatin interactions in adult EryC (38,41). Here, we show that PIC components are normally recruited to HS3 in Δ2B HbA^+ EryC but not in Δ2B e10.5 EryC. Based on this, and supported by a previous literature report (41), we propose that HS3, in the absence of HS2, is sufficient to enhance huβ-gene transcription, although it is unable to favor proper PIC formation at huγ-promoters.

Finally, we observed that chromatin organization at the β-globin locus is very similar in ln2 and Δ2B HbA^+ EryC, suggesting that chromatin in both cell types is favorable for gene expression. However, chromatin alterations were detected at the huγ-region in Δ2B HbA^+ EryC (Figure 7B and C). Since such alterations correlate with abnormal PIC formation at huγ-promoters (Figure 7D and E), we propose that chromatin organization at globin gene regions (74) is defined by LCR-facilitated recruitment/stability of *trans*-acting factors in direct contact with these regions, as well as by locus wide LCR-mediated effects.

In summary, our data show that HS2 deletion in Δ2B e10.5 cells reduces the level of huγ-gene transcription, even though the β-globin locus maintains normal chromatin organization. Moreover, other regions of the LCR cannot compensate for loss of HS2 enhancer activity, which is required in both e10.5 yolk sac and e12.5 fetal liver EryC for high-level expression of the huε- (at e10.5) and huγ-genes (at e10.5 and e12.5), but not of the huβ-gene. Thus, LCR enhancer activity cannot prevent abnormal globin gene expression due to alteration of chromatin organization. Based on the mechanistic evidence provided here, we propose that deletion of HS2 modifies LCR integrity and precludes efficient long-range interactions between LCR and huγ-promoters, which in turn impairs optimal PIC formation, promoter organization and, hence, appropriate huγ-gene expression levels.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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