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An insulator blocks spreading of histone acetylation and interferes with RNA polymerase II transfer between an enhancer and gene

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ABSTRACT

We studied the mechanism by which an insulator interrupts enhancer signaling to a gene using stably replicated chromatin templates containing the human β-globin locus control region HS2 enhancer and a target globin gene. The chicken β-globin 5′ HS4 (cHS4) insulator acted as a positional enhancer blocker, inhibiting promoter remodeling and transcription activation only when placed between the enhancer and gene. Enhancer blocking by cHS4 reduced histone hyperacetylation across a zone extending from the enhancer to the gene and inhibited recruitment of CBP and p300 to HS2. Enhancer blocking also led to accumulation of RNA polymerase II at HS2 and within cHS4, accompanied by its diminution at the gene promoter. The enhancer blocking effects were completely attributable to the CTCF binding site in cHS4. These findings provide experimental evidence for the involvement of spreading in establishment of a broad zone of histone modification by an enhancer, as well as for blocking by an insulator of the transfer of RNA polymerase II from an enhancer to a promoter.

INTRODUCTION

Similar to upstream activating sequences in yeast, enhancers recruit chromatin remodeling complexes and RNA polymerase II (pol II) through the action of sequence-specific DNA binding factors (1,2). As a result, remodeling complexes act at distant target promoters to create a structure amenable to transcription complex formation. Remodeling complexes fall primarily into two groups. Nucleosome remodeling complexes of the SWI/SNF type (3) use the energy of ATP hydrolysis to alter nucleosome structure and/or position, while other complexes covalently modify the N-terminal tails of histones by acetylation, methylation, phosphorylation and ubiquitinylation (4).

Several models have been proposed to explain the mechanism of transfer of activation components from enhancers to promoters (5–7). Consistent with a looping or direct contact model, pol II and histone acetylation are detected at the enhancer and promoter of the prostate-specific antigen (PSA) gene, but not in the intervening sequences (8). Similarly, in the TCRβ locus, the histone acetyltransferase (HAT) CBP and pol II were associated with the promoter and enhancer (9). Alternatively, a report that pol II is recruited to the PSA enhancer independent of recruitment to the promoter, and is detected (in contrast to the findings noted above) along with histone acetylation between enhancer and promoter supports a tracking model (10). The facilitated tracking model proposes that activators track along the DNA from enhancer to promoter, but retain enhancer contact, eventually forming a loop (11). Activators, HATs and the SWI/SNF component Brg1, are detected in such a fashion at and between an enhancer and the HNF-4α promoter, although pol II was only detected at the promoter (12).

The β-globin locus control region (LCR) illustrates a more complex enhancer organization than the above examples. It contains four DNase-I-hypersensitive regions (HS1-4) to which bind clusters of transcription factors, including the erythroid factor NF-E2 (13). The functional genes of the locus, ε, $^{G}\gamma$, $^{A}\gamma$, δ and β, are expressed at different developmental stages, and are located 6–60 kb downstream of the LCR, which is required for their high level transcription (14). Novel approaches have recently provided direct evidence in endogenous globin loci for physical proximity between the LCR and the actively transcribing β-globin genes (15–17), solidifying a role for looping in LCR-promoter communication. Further, pol II was associated with the murine LCR HSs and active β-globin promoters, but not the intervening, inactive embryonic promoters, a result compatible with looping (18–21). However, the existence of long intergenic pol II transcripts and extensive domains of histone hyperacetylation within the β-globin locus imply that tracking may be a component of LCR activity (22–24).

Insulators are nucleoprotein structures which are chromatin boundary elements and can also act as enhancer blockers, assuring that an enhancer will only interact with an appropriate gene target (25). The best characterized insulators include the *Drosophila* gypsy retrotransposon and specialized chromatin structure (scs) elements, and chicken β-globin 5′ HS4 (cHS4), each of which has an associated protein, Su(Hw), Zw5 and CTCF, respectively, to which enhancer blocking is attributed (26–28). Enhancer blocking occurs when an insulator is positioned between an enhancer and promoter, without apparently compromising the ability of either to communicate with elements from which they are not insulated (29,30). An insulator

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might physically block the tracking of complexes between an enhancer and promoter. Alternatively, an insulator could prevent looping either directly by acting as a 'decoy', or otherwise indirectly impair enhancer function (31,32).

Clearly, the functions of enhancers and insulators are intertwined. Since enhancer blocking assays measure transcription output or protection from position effect variegation, little is known about the effect of enhancer blocking on the modulation of chromatin structure which is associated with enhancer activation of genes. Here we have investigated how the signal from an enhancer to a promoter is interrupted by an insulator using a model system in which chromatin structural changes attributable to the enhancer are well defined (33,34). We found that positional enhancer blocking by cHS4 impaired the ability of the β-globin LCR HS2 enhancer to remodel the human embryonic ε-globin gene promoter chromatin and to activate transcription. Enhancer blocking also resulted in a widespread decrease in histone acetylation between the insulator and gene, and within the gene. Finally, the interposition of cHS4 between the enhancer and gene led to redistribution of pol II in the locus with accumulation of pol II at HS2 and in cHS4, and reduced detection at the gene promoter. We discuss these results in the context of models of enhancer and insulator action.

MATERIALS AND METHODS

Minichromosome construction, cell culture conditions and transfection

Minichromosomes carrying the ε-globin gene (3.7 kb EcoRI fragment) and HS2 (374 bp HindIII to XbaI fragment) have been described previously (33). The 1.2 kb cHS4 insulator was cut from plasmid pJC5-4 (a kind gift of G. Felsenfeld) with XbaI (35) and inserted into pGEMHS2ε to create cHS4in, cHS4out and cHS4both. Clone PCI (ΔII) containing the cHS4 core 250 bp element with a 23 bp deletion eliminating the CTCF site was a kind gift of A. West. Details of swapping this fragment into cHS4in are available upon request. Drosophila scs (1.8 kb) was cut from a plasmid (a kind gift of R. Kellum) with XbaI, and similarly inserted into pGEMHS2ε. The constructs were cloned into EBV-based minichromosomes which are stably maintained at 10–20 copies/cell in K562. Transfection conditions, growth of K562 cells and isolation of individual clones carrying minichromosomes have been described in (33,36). Between 12 and 15 clones of each type were tested for ε-globin transcription. Chromatin structure was studied for 2 or 3 representative clones and 1 or 2 clones of each type were selected for chromatin immunoprecipitation (ChIP).

RNase protection assay

RNA was prepared from 5×10^7 cells of K562 clones carrying minichromosomes, and RNase digestion and gel analysis were performed as recommended by the manufacturer (Ambion). The RNA probe used was generated with T7 polymerase from NcoI-linearized pBS458 (33), which contains an EcoRV–SspI fragment from the ε-globin gene promoter and the 5′ flanking region. The intensity of bands was quantified on a PhosphorImager (Molecular Dynamics) using the ImageQuant software, and the results normalized to the actin control signal. Endogenous ε-globin signals typically vary among K562 cell clones but are unrelated to minichromosome transcription.

Preparation of nuclei and nuclease treatment

Nuclei of K562 cell clones (1×10^8 to 1.5×10^8 cells) were suspended in 0.3–0.5 ml of wash buffer and digested with 0, 6 or 12 μg/ml of DNase I for 10 min at room temperature in the presence of 3 mM $CaCl_2$ (33). Alternatively, nuclei were suspended to 360 μl in wash buffer, and 60 μl aliquots were digested with 100 U of restriction enzymes in a final volume of 400 μl of appropriate buffer (New England Biolabs), conditions previously determined to yield maximal digestion (36). Purified DNA was cut to completion with a different enzyme and subjected to gel electrophoresis and Southern blotting. Blots were hybridized with probes labeled by random priming to a specific activity of 1×10^9 to 2×10^9 c.p.m./μg of DNA, and the intensity of bands was quantified with a PhosphorImager.

Chromatin immunoprecipitation

Histone modifications were studied by ChIP as described previously (37). Briefly, nuclei from 5×10^7 cells were divided into three aliquots which were digested with increasing concentrations of MNase at 37°C for 10 min. The digests were combined and passed over a 5–30% sucrose gradient, and the mono- and di-nucleosome-containing fractions were retained. Chromatin was pre-cleared by incubation with protein A–agarose, and a sample retained for preparation of the 'input' sample. DNA was purified from specifically immunoprecipitated chromatin and from the reserved 'input' sample and their concentrations determined using picogreen fluorescence (Molecular Probes, Eugene, OR).

For analysis of pol II, NF-E2 and CTCF, 2.5×10^7 cells were first cross-linked with 0.4% formaldehyde for 10 min at room temperature and ChIP was performed as described, using sonication to fragment the chromatin (38). Chromatin was precleared by incubation with protein A–agarose, and a sample retained for preparation of the 'input' sample. After reversal of cross-links, DNA was purified from specifically immunoprecipitated chromatin and from the reserved 'input' sample. ChIPs using antibodies to CBP and p300 were carried by similar methods except cells were cross-linked with 1% formaldehyde (39). After cross-linking, nuclei were prepared and incubated with 200 U of MNase for 15 min at 37°C. MNase digestion was stopped by adding EDTA to a final concentration of 10 mM, and the chromatin was then sonicated.

Antibodies

Anti-acH3 (06-599), anti-acH4 (06-866) and anti-CTCF (06-917) were purchased from Upstate Biotechnology, Lake Placid, NY. Anti-NF-E2 (sc-291), anti-pol II (sc-899), anti CBP (sc-369) and anti-p300 (sc-8981) were purchased from Santa Cruz Biotechnology, Santa Cruz, CA.

Quantitative real-time PCR and data analysis

Differences in DNA enrichment for histone ChIP samples were determined by real-time PCR on 1 ng samples of DNA using the ABI Prism 7900 (PE Applied Biosystems)

Table 1. TaqMan probes and primers

Target area	Primer or probe
cHS4/CTCF	Forward 5′-AGC CCC CAG GGA TGT AAT TA
	Reverse 5′-CGG ACC GGA GCG GAG
	Probe 5′-6FAM-CAG CAG CGA GCC GCC C-TAMRA
cHS4 middle	Forward 5′-CAG GAC AGC ATG GAC GTG G
	Reverse 5′-TTC TGA ACG CTG TGA CTT GGA
	Probe 5′-6FAM-CAT GCA GGT GTT GAG GCT CTG GAC A-TAMRA
cHS4/ΔII	Forward 5′-CCA AAG CCC CCA GGG AT
	Reverse 5′-CGG ACC GGA GCG GAG
	Probe 5′-6FAM-AGC AGC GAG CCG CCC GG-TAMRA
β-globin exon 2	Forward 5′-TGG GCA ACC CTA AGG TGA AG
	Reverse 5′-GTG AGC CAG GCC ATC ACT AAA
	Probe 5′-6FAM-TCA TGG CAA GAA AGT GCT CGG TGC-TAMRA
Necdin	Forward 5′-TTC GTC CAA ATG AAT TAC CTG AAG
	Reverse 5′-GGA GCC CCA AAA GAA CTC GTA
	Probe 5′-6FAM-ACC AGC GCG TCC CAT ACG TGG AG-TAMRA

(37). The threshold was set to cross a point at which PCR amplification was linear, and the number of cycles (Ct) required to reach the threshold was collected and analyzed using Microsoft Excel. The fold difference of a given target sequence precipitated by the anti-histone antibodies was determined by dividing the amount of target sequence in the immunoprecipitated fraction by the amount of target sequence in input DNA. The relative enrichment of a given target sequence was then obtained by normalizing the fold difference of the sequence by the fold difference obtained for a primer in the endogenous β-globin gene. A similar analysis was carried out for the formaldehyde cross-linked chromatin samples immunoprecipitated by antibodies to pol II, NF-E2, CTCF, CBP and p300 except that 2.5% of the precipitated sample DNA and 0.02% of the input DNA were used in real-time PCR. The brain specific necdin gene served as a control.

Primers and TaqMan probes

Primers and TaqMan probes selected using PE Applied Biosystems Primer Express software, were obtained from Invitrogen and PE Applied Biosystems, respectively. Amplicons were designed to be <147 bp with an average size of 86 bp. The sequences of the primers and TaqMan probes are given in Table 1 or have been described previously (34).

RESULTS

The cHS4 insulator impairs HS2 dependent ε-globin gene transcription activation

On stably maintained minichromosomes, transcription of the human ε-globin gene is activated >100-fold by the β-globin μLCR, a fusion of HS1–4, and equivalently by the strong HS2 enhancer alone (33). To study the interplay between an enhancer and its target gene, the cHS4 insulator and the *Drosophila* scs insulator were each cloned either outside an HS2/ε-globin model locus, between the enhancer and the gene, or in both positions (Figure 1A). The minichromosomes were stably maintained in human erythroid K562 cells in which the endogenous ε-globin gene is actively transcribed. RNase protection assays were performed to test the influence of the insulators on enhancer activation of the ε-globin gene. The assay distinguishes episomal from endogenous ε-globin transcripts because the episomal copy is marked by a mutation in

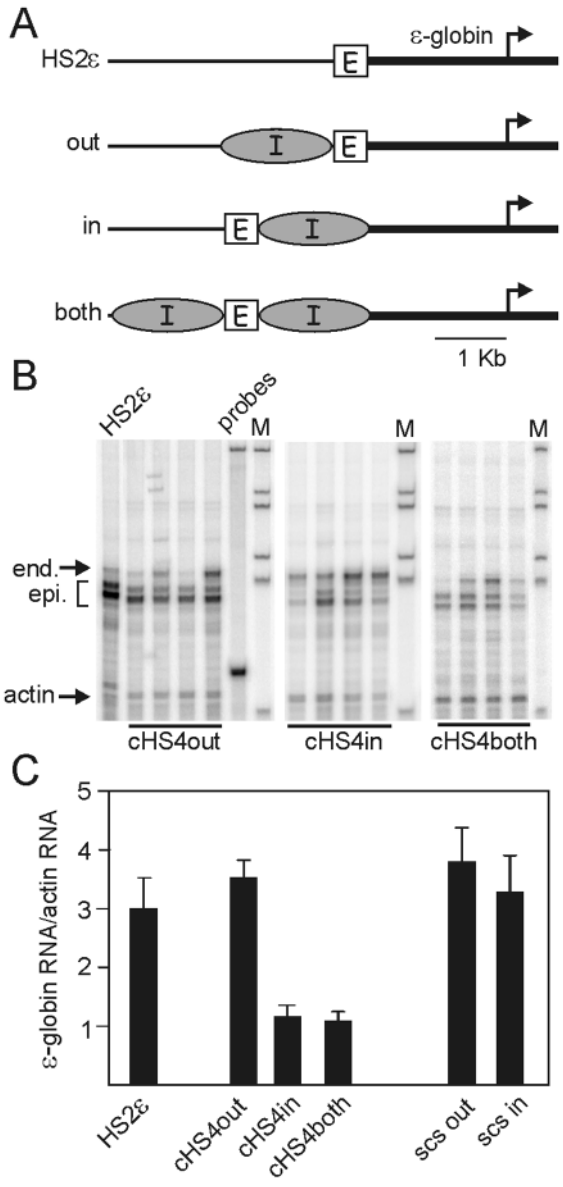

Figure 1. HS2-activated transcription of the ε-globin gene is blocked by the cHS4 insulator. (**A**) The β-globin LCR HS2 enhancer and ε-globin gene form a model globin gene locus in which the enhancer lies at a distance of 2 kb from the target gene. The cHS4 and scs insulators were cloned either outside (out) the enhancer–gene unit, between the enhancer and the gene (in), or on both sides of the enhancer (both). (**B**) RNA was isolated from clones of cells carrying the different minichromosomes, and endogenous (end.) and episomal (epi.) ε-globin RNA was detected by RNase protection. Note that endogenous ε-globin signals typically vary among K562 cell clones for unknown reasons which do not affect minichromosome transcription. Representative results for four clones of each type are presented. M, DNA size markers. (**C**) The mean ε-globin RNA normalized to actin RNA (±SEM) for 12–15 clones of each type is illustrated graphically.

the 5′-untranslated region resulting in shorter protected fragments than are produced by endogenous transcripts.

Representative assays of RNA from different cHS4 insulator-containing clones are illustrated in Figure 1B, and the results for least 12 individual clones of each type are summarized in Figure 1C. Abundant transcription of the ε-globin RNA occurred when the gene was linked to HS2 (HS2ε, Figure 1B). Transcription was compromised by cHS4 in a positional manner, i.e. only when it was between the enhancer and the gene (cHS4in, cHS4both, Figure 1B and C). In contrast to cHS4, *Drosophila* scs was unable to block the enhancer in either position (scs out, scs in, Figure 1C, and data not shown). The size of the scs element (1.8 kb) is larger than cHS4 (1.2 kb), which argues that the inhibition of transcription by interposed cHS4 is not due to an increase in distance between HS2 and the ε-globin gene, but due to a specific effect of cHS4.

Placing cHS4 outside the enhancer–gene unit had no effect on transcription, indicating that enhancer blocking was not due to formation of a broad domain of silenced chromatin. Furthermore, when the insulator flanked the enhancer on both sides (cHS4both, Figure 1C), inhibition of transcription was only slightly stronger than with only the intervening insulator. We conclude that on replicating minichromosomes, positional enhancer blocking by cHS4 is maintained as in a chromosomal context (35), consistent with other studies using EBV-based episomes (40). Positional enhancer blocking implies a processive mechanism underlying enhancer action.

cHS4 inhibits chromatin remodeling of the ε-globin gene by HS2

Enhancer signaling to promoters results in SWI/SNF-type chromatin remodeling which alters the structure of the target gene so that activators and components of the transcriptional machinery can access the promoter. However, enhancer blocking is typically measured by reduction of transcriptional output or protection from position effect variegation; the detailed chromatin structure of insulated promoters and enhancers has not been studied extensively. Chromatin structure changes associated with transcriptional activation of ε-globin by HS2 on minichromosomes are known in detail (33,34). Therefore we asked whether enhancer blocking by cHS4 affected enhancer dependent chromatin remodeling using this system.

The promoter of the active ε-globin gene forms a discrete DNase-I-hypersensitive site on minichromosomes as it does in the endogenous β-globin locus in K562 cells (36). This site was analyzed in the nuclei of insulator containing clones. Formation of the DNase I HS was unaffected when cHS4 was outside the HS2 enhancer–gene unit (Figure 2A, compare HS2ε with cHS4out). However, hypersensitivity at the promoter was decreased when cHS4 was interposed between the enhancer and gene (Figure 2A, cHS4in) where it blocks transcription activation. A more quantitative assessment of chromatin remodeling was obtained by investigating AvaII accessibility in the ε-globin promoter. A representative experiment is shown in Figure 2B, and the results summarized (±SEM) in Figure 2C. AvaII accessibility in the ε-globin promoter decreased ~35%, when cHS4 was between the enhancer and gene ($P < 0.05$ using the unpaired t-test), consistent with the results in Figure 2A. However, when cHS4 was outside the enhancer–gene unit there was no significant effect on promoter chromatin structure.

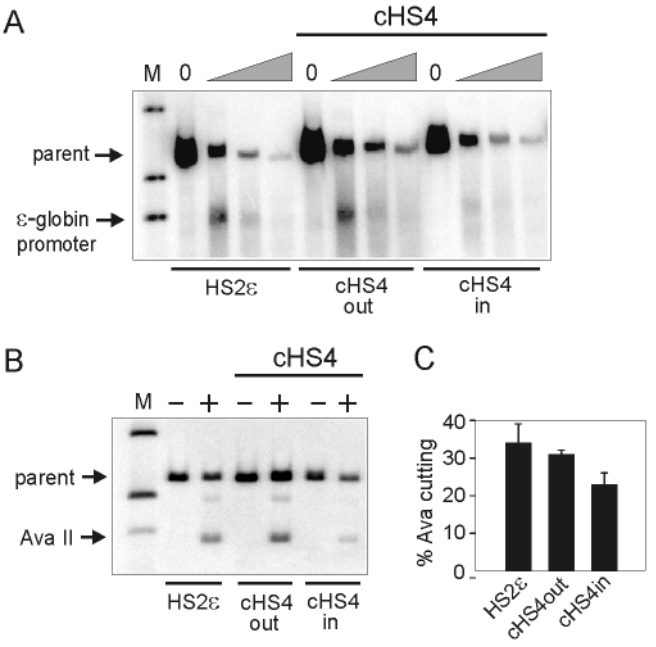

Figure 2. Formation of the ε-globin promoter DNase-I-hypersensitive site is reduced by cHS4. (**A**) DNase I hypersensitivity of the ε-globin promoter was tested by indirect end-labeling on two or three clones of each type. Nuclei from K562 cell clones carrying uninsulated (HS2ε) and insulated loci (cHS4out, cHS4in) were incubated with varying amounts of DNase I. DNA was then purified and cut to completion with BglII to yield a parent fragment. Representative results are depicted. M, molecular weight markers. (**B**) Restriction enzyme accessibility was tested in nuclei of clones carrying uninsulated (HS2ε) and insulated loci (cHS4out, cHS4in). Nuclei were either mock digested (−) or digested with AvaII. Purified DNA was subsequently cut to completion with a different enzyme to yield a parent fragment. Representative results are depicted. M, DNA size markers. (**C**) The intensity of bands was quantified using a PhosphorImager. The results of three determinations of the percentage restriction enzyme cutting [cut/(cut + uncut)] in the promoter for between 2 and 6 clones of each type (±SEM) are depicted.

The HS2 enhancer comprises a strong DNase-I-hypersensitive site (Figure 3A, HS2ε). This site forms whether the cHS4 insulator is outside or inside the enhancer–gene unit, although the kinetics of DNase I cleavage were somewhat altered for cHS4in (Figure 3A, cHS4out, cHS4in). Interestingly, the DNase-I-sensitive site formed by cHS4 itself (41) was also detected when it was positioned outside HS2 (Figure 3A, cHS4out asterisk). An 'inside' copy of cHS4 cannot be detected because of a virtually complete DNase I cleavage of the HS2 site between the probe and cHS4. Accessibility of HS2 sequences to PpuMI cleavage was then assessed. Figure 3B depicts a representative experiment, and the data are summarized graphically in Figure 3C. Restriction enzyme accessibility at the PpuMI site was not significantly altered by the insulator in either position (cHS4in accessibility decreased <10% and was not statistically significant). We conclude that the positional enhancer blocking by cHS4 involves compromising the ability of an enhancer to remodel a promoter. Enhancer blocking did not have major effects on enhancer chromatin structure.

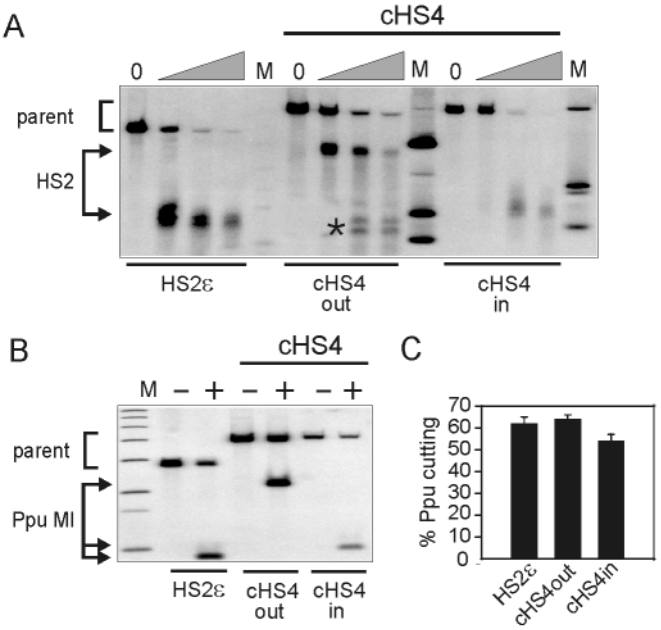

Figure 3. The cHS4 insulator has little effect on HS2 enhancer structure. (**A**) DNase I hypersensitivity at HS2 in uninsulated (HS2ε) and at HS2 and cHS4 in insulated loci (cHS4out, cHS4in) was tested by indirect end-labeling as detailed in the legend to Figure 2. Note that the EcoRV-generated parent bands and HS bands differ in size among the constructs due to the absence/presence of cHS4 and its position. M, DNA size markers. (**B**) Restriction enzyme accessibility was tested by PpuMI digestion in nuclei from clones carrying uninsulated (HS2ε) and insulated loci (cHS4out, cHS4in) as detailed in the legend to Figure 2. Representative results are depicted. M, DNA size markers. (**C**) The intensity of bands was quantified using a PhosphorImager. The results of three determinations of the per cent restriction enzyme cutting [cut/(cut + uncut)] in HS2 for between 2 and 6 clones of each type (±SEM) are depicted.

Histone acetylation across the locus is inhibited by the cHS4 insulator

Histone H3 and H4 acetylation is linked to gene expression, and is a component of enhancer-mediated transcription activation. On HS2ε minichromosomes, the HS2 enhancer-mediated, wide-spread H3 and H4 acetylation from HS2 to the ε-globin gene at a distance of 2 kb, similar to the effect of the IgH LCR on the c-myc gene (34,42). We used ChIP to investigate the effect of the cHS4 insulator on HS2-mediated histone acetylation. To produce a high resolution analysis, mono- and di-nucleosome-sized chromatin was purified following MNase digestion of nuclei from insulator containing clones (see Supplementary Figure 1A) and reacted with antibodies to acH3 and acH4. The input DNA and the immunoprecipitated DNA were amplified by quantitative real-time PCR using TaqMan probes whose positions within the insulated loci are shown in Figure 4A.

The ε-globin gene in HS2ε is hyperacetylated on H3 and H4 compared to the gene when linked to an inactivated enhancer (34). This pattern was unaltered by cHS4 outside the enhancer–gene unit (cHS4out, Figure 4B and C). In contrast, interposing cHS4 between the enhancer and gene decreased acH3 and acH4 in the ε-globin gene and, importantly, between the insulator and the gene (cHS4in, Figure 4B and C). This result suggests that the insulator acts as a block to spreading of acetylation from the enhancer to the gene. Interestingly, when cHS4 was between HS2 and the target gene, a further increase in the normally elevated levels of acH3 and acH4 at the cHS4 CTCF site was observed [(37), Figure 4B and C]. These results are consistent with recruitment of HAT activity by HS2 and spreading of acetylation toward the gene.

Recruitment of CBP and p300 to HS2 is diminished by cHS4

The HATs CBP and p300 play important roles in hematopoietic differentiation (43). Furthermore, CBP is detected at HS3 of the β-globin LCR in murine MEL cells by chromatin immunoprecipitation (44). In addition, transient assays revealed that the enhancer activity of HS2 on a γ-globin promoter driving luciferase is sensitive to the CBP/p300 antagonist E1A (45). We have observed that the same is true for luciferase under the control of the ε-globin promoter (C. Gui and A. Dean, unpublished data), suggesting that the CBP/p300 acetyltransferases are important to HS2-mediated ε-globin transcription.

We asked whether CBP and p300 could be detected in the model HS2/ε-globin locus by performing ChIP assays on uninsulated and insulator-containing clones using anti-CBP and anti-p300 antibodies after cross-linking with 1% formaldehyde and fragmentation of chromatin by MNase digestion and sonication (see Supplementary Figure 1B). The probes used were the same as illustrated in Figure 4A. Both CBP and p300 were strongly cross-linked to the HS2 enhancer in an uninsulated HS2ε locus (Figure 5A and B). Surprisingly, the HATs were not detected at significant levels elsewhere in the locus even though the domain of acetylated histones in HS2ε extends from the enhancer through the gene at a distance of 2 kb (see Figure 4). Possible reasons underlying this lack of cross-linking are discussed below. We conclude that CBP and p300 are recruited to HS2.

We found that cHS4 reduced recruitment of p300 when interposed between the enhancer and promoter, but not when situated outside the enhancer–gene unit (Figure 5A, cHS4in, cHS4out). This suggests that p300 is involved in the spreading of histone acetylation between enhancer and promoter which is diminished by positional enhancer blocking. In contrast, CBP recruitment to HS2 was diminished when cHS4 was either inside or outside the enhancer–gene unit (Figure 5B, cHS4in, cHS4out). Thus, CBP levels at HS2 do not seem to be directly related to transcriptional enhancer blocking in this system.

CTCF and NF-E2 are associated uniquely with cHS4 and HS2 respectively

If a direct interaction of cHS4 and HS2 were required for enhancer blocking, we predicted that cross-linking and ChIP assays might reveal an enhancer factor associated with the insulator, and vise versa, due to protein–protein interactions. Therefore, we used antibodies to the HS2 enhancer factor NF-E2 and the insulator factor CTCF in ChIP assays of the cHS4in and cHS4out insulated loci. After cross-linking with 0.4% formaldehyde, the chromatin was sonicated to 100–500 bp fragments (see Supplementary Figure 1C) and

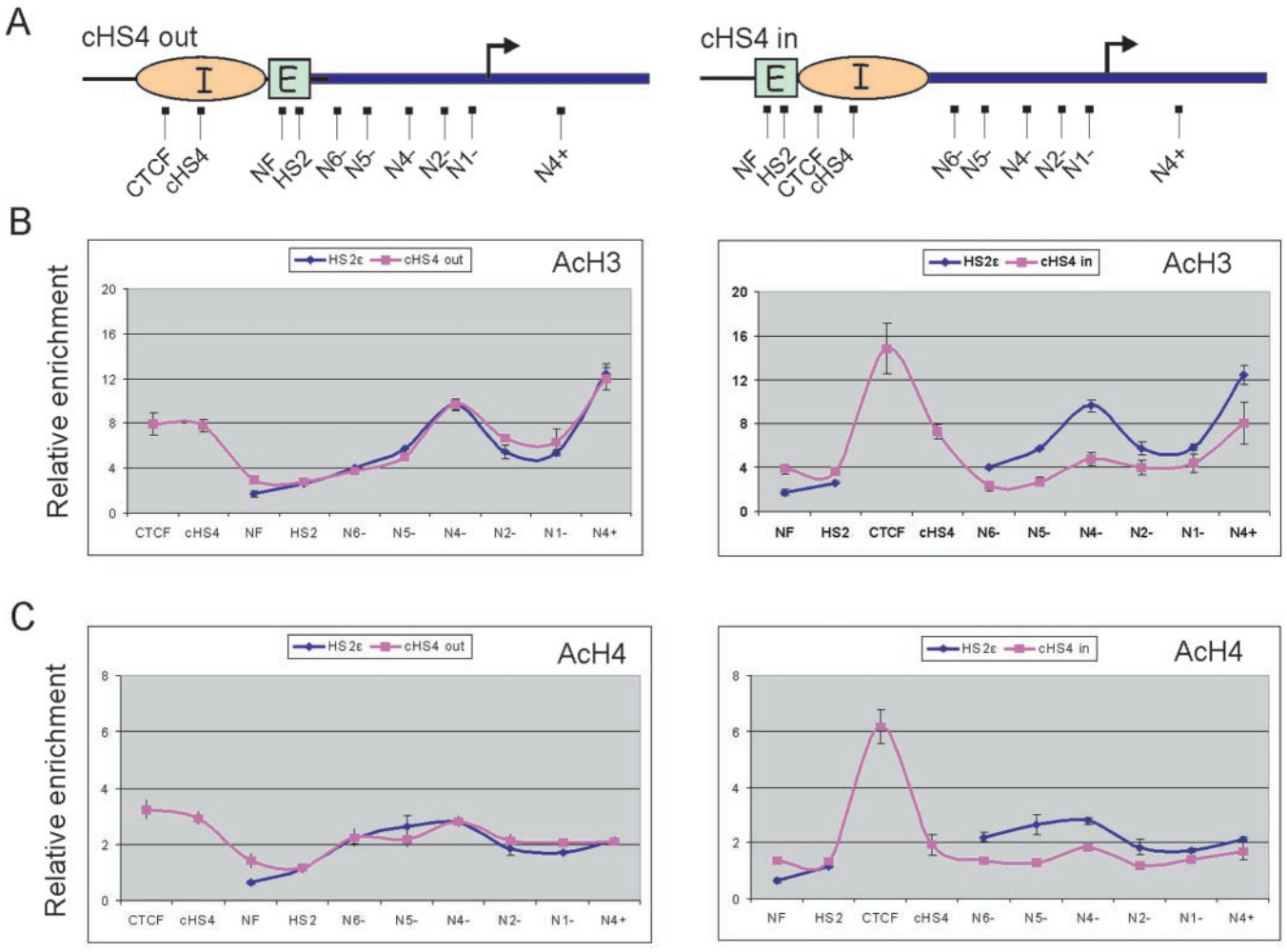

Figure 4. cHS4 alters histone acetylation in a positional manner. (**A**) The positions of TaqMan primers used for real time PCR are indicated beneath the cHS4out and cHS4in constructs. Primer NF amplifies the NF-E2 site region of HS2 while primer HS2 amplifies sequences about 150 bp 3′ to this site. N6– to N4+ correspond to actual or inferred nucleosome positions relative to the ε-globin promoter (33). N4+ lies within the ε-globin second intron [see Table 1 and (34) for sequences]. (**B**) Chromatin was prepared from nuclei of K562 cells carrying uninsulated (HS2ε) and insulated loci (cHS4in, cHS4out) using MNase and ChIP was then carried out using antibodies to acH3. The fold difference and relative enrichment for each primer pair was determined (see Materials and Methods). The results for at least three chromatin preparations each assayed in duplicate or triplicate (±SEM) are presented. (**C**) ChIP was carried out with antibodies to acH4 as described for (B).

ChIP assays were performed using the probes illustrated in Figure 4A.

NF-E2 was associated primarily at HS2 and CTCF was associated with cHS4 in the cHS4out locus (Figure 6A). However, when cHS4 was interposed between the enhancer and gene where enhancer blocking occurred, increased NF-E2 (∼5-fold) was detected with the CTCF probe (Figure 6B). Reciprocally, there was a similar increase in the detection of CTCF with the HS2 probe when cHS4 was interposed between the enhancer and gene.

To rule out the possibility that the association of CTCF with HS2 and of NF-E2 with cHS4 resulted from the proximity of the amplified sequences (<250 bp) rather than an interaction underlying enhancer blocking, we reversed the direction of the cHS4 insulator (cHS4inR), increasing the separation between the HS2 NF-E2 site and the cHS4 CTCF site, which lies at the 5′ end of cHS4, to 1.3 kb. Enhancer blocking of transcription and histone acetylation were unaffected by reversal of the direction of the insulator as expected (46) (data not shown). However, CTCF detection in HS2 and NF-E2 detection at the CTCF site were low (Figure 6C, cHS4inR), indicating that these associations are not required for enhancer blocking and probably result from the proximity of the respective amplicons. Thus, direct interaction of the enhancer and insulator through interaction of CTCF and NF-E2 could not be demonstrated using these approaches.

The cHS4 insulator alters the localization of pol II in the locus

Considerable evidence supports the idea that enhancers recruit pol II, and, in some cases, that pol II is transferred to a promoter from an enhancer by loop formation between the two elements (8,9). In other experiments pol II was detected between the enhancer and promoter, consistent with movement along the chromatin to the promoter (10). To investigate whether enhancer blocking by cHS4 perturbed pol II distribution in the model locus, as it had histone acetylation,

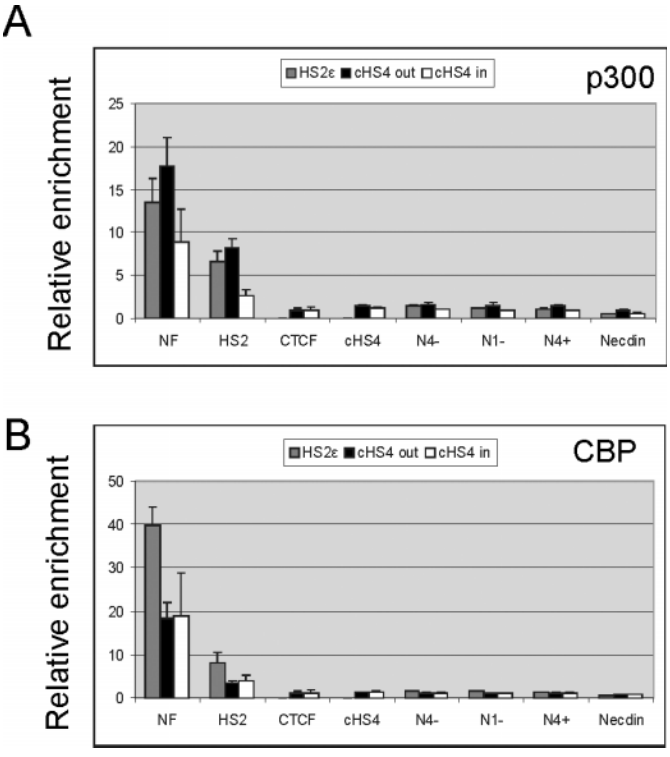

Figure 5. CBP and p300 recruitment is diminished by cHS4. (**A**) Chromatin was prepared from 1% formaldehyde cross-linked K562 cells carrying uninsulated (HS2ε) and insulated (cHS4in, cHS4out) loci, and MNase digested and sonicated chromatin was subjected to ChIP using antibodies to p300. (**B**) ChIP was carried using uninsulated HS2ε and insulated cHS4in and cHS4out with antibodies to CBP. The fold difference and the relative enrichment for each primer pair were determined. The results for at least three chromatin preparations assayed in duplicate or triplicate (±SEM) are presented. Probes were the same as for Figure 4.

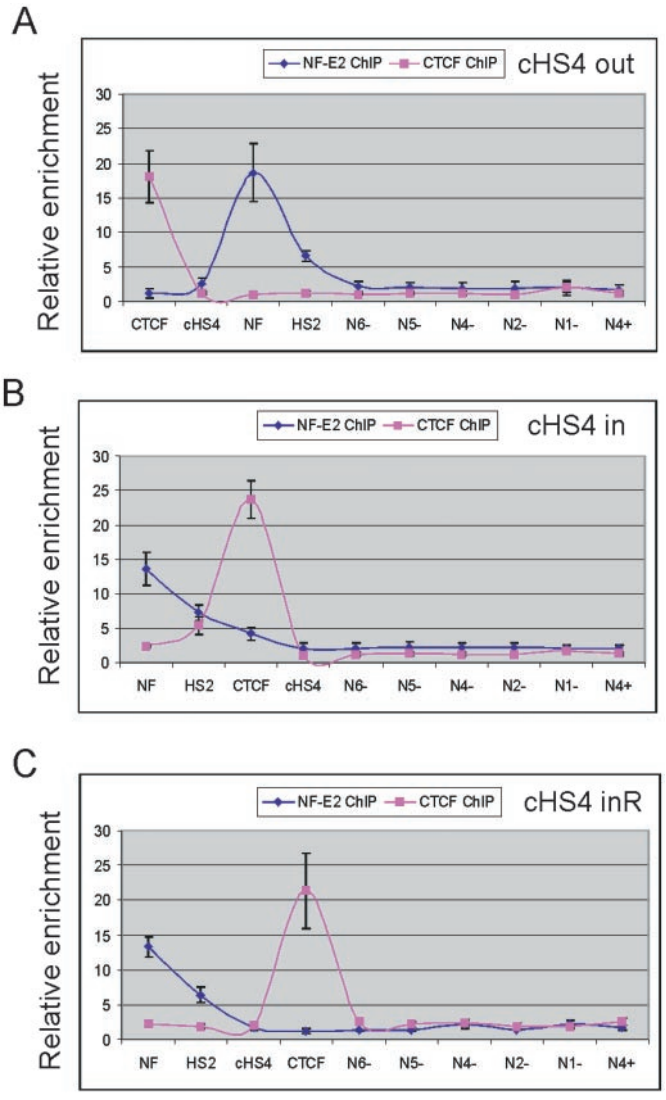

Figure 6. NF-E2 and CTCF localization within uninsulated and insulated loci. (**A**) Chromatin was prepared from 0.4% formaldehyde cross-linked K562 cells carrying cHS4out and was sonicated prior to ChIP using antibodies to NF-E2 and CTCF. The fold difference and the relative enrichment for each primer pair was determined. The results for at least three chromatin preparations assayed in duplicate or triplicate (±SEM) are presented. Probes were the same as for Figure 4. (**B**) ChIP was carried out as described for panel A using cHS4in. (**C**) The cHS4 insulator was reversed in the context of cHS4in to create cHS4inR and ChIP experiments were carried out as described for (A).

we performed ChIP on insulator-containing clones using anti-pol II antibodies after cross-linking with 0.4% formaldehyde and sonication of chromatin to 100–500 bp fragments (see Supplementary Figure 1C).

In HS2ε, pol II was detected strongly at the ε-globin promoter (N1−) and within the gene coding sequences (N4+), and weakly within HS2 and between the enhancer and promoter (Figure 7A, HS2ε). Placing cHS4 outside HS2 did not perturb this pattern (Figure 7A, cHS4out). A striking finding is that interposing cHS4 between the enhancer and gene leads to redistribution of pol II in the locus (Figure 7B, cHS4in), with a 50% decrease at the promoter and coding sequences, consistent with decreased transcription, and a 3-fold increase in the pol II signal in HS2 ($P < 0.05$). In addition, a strong pol II signal was detected at the CTCF site in cHS4. This result suggests that pol II is normally transferred from HS2 to the linked globin gene, but that cHS4 blocks this process.

The role of CTCF in enhancer blocking by cHS4 in chromatin

Enhancer blocking by cHS4 is attributable to the CTCF site therein (28). To investigate whether the effects on chromatin structure and on pol II distribution which we observed were the result of authentic enhancer blocking and thus dependent on CTCF, we created a cHS4in locus (cHS4Δ II) containing a 23 bp deletion eliminating the CTCF binding site (28). The CTCF deletion restored transcription of the ε-globin gene to uninsulated levels, consistent with the critical role of CTCF in enhancer blocking by cHS4 (Figure 8A). This data also supports our contention that reduced ε-globin transcription caused by an intact cHS4 is not due to an increased distance between the HS2 enhancer and the gene promoter.

As expected, CTCF, which is strongly detected at its site in cHS4in, was no longer detected in cHS4ΔII (Figure 8B). Deletion of the CTCF site restored histone H3 and H4 acetylation levels in the locus (Figure 8C AcH4 not shown), consistent with the idea that the insulator block to processive histone acetylation is dependent on CTCF. Interestingly, CTCF

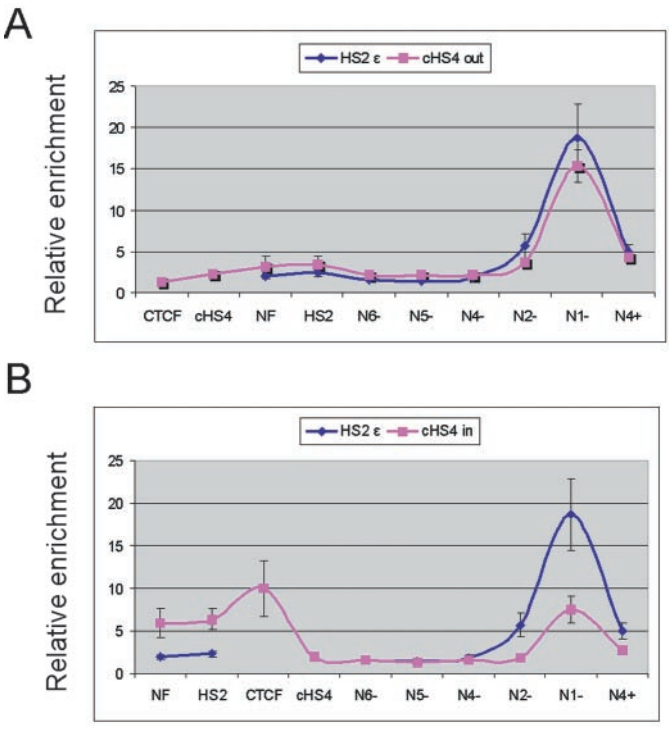

Figure 7. Pol II accumulates in HS2 and cHS4 when transcription is blocked by cHS4. (**A**) Chromatin was prepared from 0.4% formaldehyde cross-linked K562 cells carrying uninsulated (HS2ε) or cHS4 out insulated loci as described in Figure 6A. ChIP was carried out using antibodies to pol II. The fold difference and the relative enrichment for each primer pair were determined. The results for at least three chromatin preparations assayed in duplicate or triplicate (±SEM) are presented. Probes were the same as for Figure 4. (**B**) ChIP was carried out using antibodies to pol II and chromatin from cHS4 in insulated loci and compared to the results for uninsulated HS2ε as shown in (A).

deletion did not diminish the peak of histone acetylation observed in cHS4 when it is interposed between HS2 and the ε-globin gene.

The CTCF site deletion completely reversed accumulation of pol II within HS2 and cHS4, and partially restored pol II levels at the promoter consistent with the restoration of transcription (Figure 8D). The remaining footprinted regions in cHS4 are intact in this construct and are occupied by the proteins present in K562 cell nuclear extracts (47), providing an internal control for the specificity of CTCF-mediated enhancer blocking. Taken together, these experiments indicate that CTCF is a critical component of cHS4 enhancer blocking, providing a block to the spreading of histone acetylation and pol II movement from an enhancer to a promoter.

DISCUSSION

The cHS4 insulator acts as a positional enhancer blocker when interposed between the β-globin LCR HS2 enhancer and the ε-globin gene in a model system. Enhancer blocking by cHS4 impairs remodeling of promoter chromatin by the enhancer, which is required for transcription activation, but does not appear to affect HS2 enhancer structure. Enhancer blocking by cHS4 diminishes recruitment of CBP and p300 to HS2, and results in a decrease in acetylated histones, which is an enhancer-mediated effect, over the gene and sequences between it and the enhancer, consistent with spreading of this modification. Enhancer blocking also results in a striking accumulation of pol II at HS2 and within the insulator, and a commensurate decrease in both at the promoter. Thus, enhancer blocking by cHS4 antagonizes multiple functions of the HS2 enhancer, and the results suggest that enhancer activity may comprise a processive, or tracking, component in addition to a looping component which is, overall, most consistent with the facilitated tracking model.

Transcriptional enhancer blocking by cHS4

We observed that cHS4 inhibited transcription activation of the ε-globin gene by HS2 in a positional manner, but that enhancer blocking was incomplete in our system. However, the level of blocking observed (60–65% in different clones) is within the range of 50–87% observed with the 1.2 kb cHS4 insulator in other studies (28,40,48). Although stronger enhancer blocking can be achieved with a multimerized 250 bp core insulator fragment (46), critical factor binding sites within the core are thus juxtaposed and are not distinguishable from one another in ChIP assays.

The fact that scs was ineffective as an enhancer blocker in our system was unexpected. When scs is placed between an enhancer and promoter and integrated into the fly genome, the enhancer-activated transcription is blocked (49). scs strongly blocked silencing by chromatin-associated repressors on episomes in human cells, while cHS4 was considerably less active in that system (40). scs was also active in *Xenopus* oocytes in a non-chromosomal context (50). However, scs exhibited silencing when studied on non-replicating episomes (51). Furthermore, other studies indicate that scs neither blocks silencing nor protects against position effects in transgenic mice (52,53). Together these and other studies reveal a range in insulator function depending on the experimental model, the basis for which is unclear (54).

Positional enhancer blocking by cHS4 in this system is accompanied by authentic characteristics of this insulator. For example, it is a focus of histone acetylation, it works equally well when reversed and its effects are dependent on CTCF (28,55). These properties, and the very well understood chromatin structure of the HS2/ε-globin locus, provided the opportunity to investigate enhancer–insulator antagonism in molecular detail.

Enhancer blocking effects on chromatin structure

Our results indicate that positional enhancer blocking by cHS4 involves compromising the ability of the HS2 enhancer to remodel ε-globin promoter chromatin (Figure 2A and B). Extensive studies of the chromatin structure of this model locus revealed the presence of stable unaltered nucleosomes between the HS2 enhancer and the ε-globin gene at a distance of 2 kb (34). Thus, the insulator may antagonize the direct or indirect transfer of SWI/SNF between the enhancer and gene promoter (56), most likely in the absence of a processive mechanism. In contrast to the insulator effects on promoter remodeling, cHS4 does not have a major effect on enhancer structure (Figure 3A and B). These data offer structural

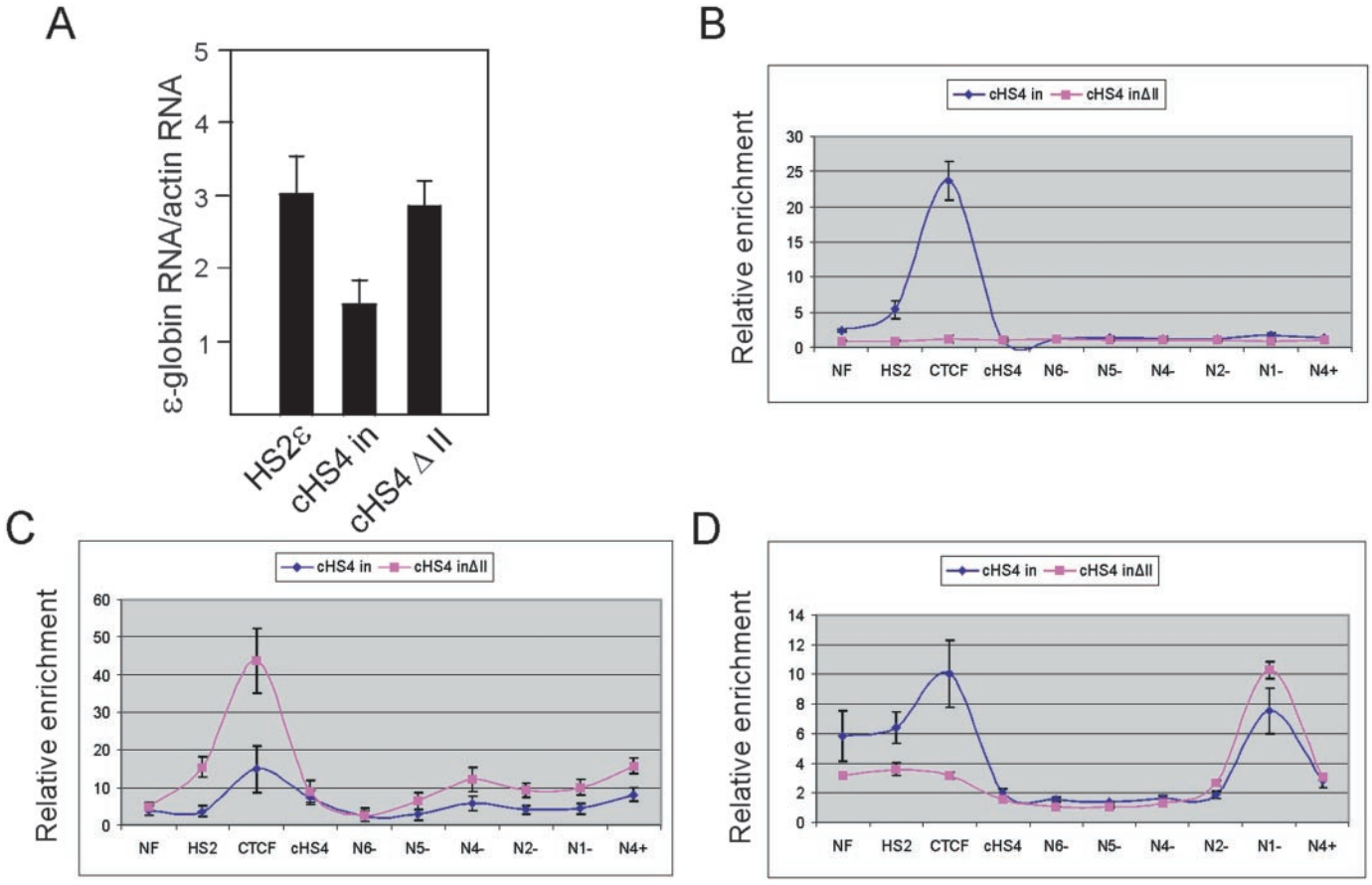

Figure 8. cHS4 blocking of histone acetylation and pol II accumulation in HS2 are CTCF dependent. (**A**) The CTCF site at the 5′ end of cHS4 was eliminated by a 23 bp deletion creating cHS4 ΔII. RNase protection assays were carried out for six clones and the average transcription (±SEM) compared to transcription in uninsulated and intact cHS4 insulated clones (HS2ε, cHS4in, see Figure 1B and C). (**B**) ChIP analysis of CTCF localization in cHS4ΔII was carried out as described in the legend to Figure 6 and the results compared to those for cHS4in. (**C**) ChIP analysis of acH3 localization were performed for cHS4Δ II as described in the legend to Figure 4 and the results compared to those for cHS4in. (**D**) ChIP analysis of pol II localization was carried out for cHS4ΔII as described in the legend to Figure 6 and the results compared to cHS4in.

support for the classic view that insulators interrupt a signal from an enhancer to a promoter, but leave the enhancer capable of activating a gene from which it is not insulated.

The HS2 enhancer mediates a domain of histone acetylation which includes the chromatin intervening between the enhancer and gene (34). Here we observed that cHS4 provides a positional block to the establishment of this domain. cHS4 also reduced recruitment of p300 to HS2 when interposed between the enhancer and promoter, but not when situated outside the enhancer–gene unit, suggesting that p300 is involved in the spreading of histone acetylation in the domain. In contrast, CBP recruitment to HS2 was diminished when cHS4 was either inside or outside the enhancer–gene unit. Thus, CBP levels at HS2 do not seem to be directly related to enhancer blocking. Perhaps CBP is involved in a function at HS2 which we have not assayed, such as acetylation of activators bound there. Diminution of the detection of HATs at HS2 could, in addition, represent a more direct effect of cHS4 on HS2 itself, such as proposed in the decoy model of insulator action (32,57).

The relatively low acetylation level observed within HS2 itself is attributable to extreme sensitivity of HS2 sequences to MNase digestion and their depletion from the input DNA (34). This sensitivity is not affected by cHS4 in any position (data not shown). In contrast, cHS4 is a focus of histone acetylation, as it is in the endogenous chicken globin locus, and becomes more highly acetylated specifically in the region of the CTCF motif when interposed between HS2 and ε-globin gene. The reason for the increase is unclear, but possibly cHS4 becomes more subject to acetylation because it is involved physically in blocking passage of an acetyltransferase. Recently, data has been presented indicating that cHS4 may be tethered to subnuclear sites via CTCF which could help in explaining how such a block could be established (58). However, deletion of the CTCF site does not diminish this peak of acetylation.

Surprisingly, CBP and p300 were not detected at substantial levels across the acetylated locus. Possibly the lack of cross-linking could be a function of HAT transit time, or a low density of HAT molecules across the locus as compared to HS2. Alternatively, HATs may not be continuously needed once a locus is modified, or may only be detectible at certain stages of the cell cycle. Another possibility is that cross-linking to specific enhancer-bound activators, such as GATA-1 and NF-E2, with which these HATs interact (59,60)

may be required for their detection using ChIPs. In any case, the data provide support for the spreading of histone acetylation from the HS2 enhancer to the ε-globin gene which is blocked by the insulator.

Enhancer blocking effects on pol II localization

Positional enhancer blocking by cHS4 resulted in accumulation of pol II at HS2 sequences and within cHS4 itself. However, in both the insulated and uninsulated loci, and similar to the result with HATs, very little detection of pol II across the locus was observed, although non-genic transcripts were consistently detected (data not shown). Possibly, the detection of pol II by ChIP at different sequences is influenced to an unknown extent by rates of procession and/or pausing and by the proximity of the protein to the DNA, concentration effects or other factors. Regardless, the insulator clearly reduced the pol II signal in the promoter and brought about a reciprocal accumulation of pol II in HS2 and within cHS4. This result suggests that at least some pol II reaches the ε-globin promoter by virtue of recruitment to the HS2 enhancer, a view consistent with observations in MEL cells and an LCR-deleted mouse globin locus (18,20).

The means of transfer of pol II between the enhancer and the gene which is interrupted by the insulator is not clear. The present data indicating only low levels of pol II detection between HS2 and the ε-globin gene in uninsulated loci would appear to argue for direct or indirect transfer of pol II from HS2 to the ε-globin gene (18), most likely by looping. In this case, an insulator might prevent the transfer of pol II by interacting with the enhancer, as proposed in the decoy model of insulator action. However, we were unable to detect interaction of CTCF with HS2 or of NF-E2 with cHS4. Intriguingly, a pattern of pol II accumulation at HS2 similar to that induced by the cHS4 insulator results subsequent to the inhibition of elongation by DRB (A. Kim and A. Dean, unpublished data) and the same effect is notable after DRB treatment of MEL cells (21). Thus, it remains a possibility that movement of pol II across sequences intervening between an enhancer and gene is functionally involved in enhancer-mediated gene activation and that such movement is blocked by cHS4.

Enhancer blocking by cHS4 and enhancer mechanisms

Several reports support the tracking of at least some components mediating transcription activation, including pol II, from enhancers to promoters (9,10,12), but other results support looping exclusively (8). In one report, the formation of a loop appears to be guided by the movement of components from the enhancer to the promoter with retention of enhancer contact (12), consistent with the facilitated tracking model (11). This mechanism could provide a rationale for loop formation, since random collision of a promoter and distant enhancer is highly energetically unfavorable.

In the present work, we studied the effect of the enhancer blocker cHS4 on the activity and chromatin structure of a model enhancer–gene locus consisting of β-globin LCR HS2 and the embryonic ε-globin gene separated by about 2 kb of normally intervening DNA. The results suggest that both looping and tracking may play a role in enhancer mediated gene activation. In the endogenous human β-globin locus, the ε-globin gene is separated from the LCR by ~6 kb, similar to the separation of the PSA, pDβ1 and HNF-4α genes from their enhancers in the examples cited above. We have observed continuous distribution of histone acetylation and pol II between the LCR and the endogenous human ε-globin gene in K562 cells (61). Furthermore, cross-linking of acetylated histones and pol II increased with increased ε-globin transcription. Taken together, these results lead us to speculate that globin gene activation by the LCR during development may also have both looping and tracking components. This proposal is consistent with the developmentally regulated domains of chromatin remodeling and intergenic transcription which have been described in a transgenic human globin locus (23). Experiments are underway to test these possibilities in a complete human globin locus.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at NAR Online.

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