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### Structural and functional conservation at the boundaries of the chicken $\beta$ -globin domain

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# Structural and functional conservation at the boundaries of the chicken $\beta$ -globin domain

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**We show that the 3' boundary of the chicken  $\beta$ -globin locus bears striking structural similarities to the 5' boundary. In erythroid cells a clear transition in DNase I sensitivity of chromatin at the 3' end of the locus is observed, the location of this transition is marked by a constitutive DNase I hypersensitive site (HS), and DNA spanning this site has the enhancer-blocking capacity of an insulator. This HS contains a binding site for the transcription factor CTCF. As in the case of the 5' insulator, the CTCF site is both necessary and sufficient for the enhancer-blocking activity of the 3' boundary. The position of this insulator is consistent with our proposal that it may function to maintain the distinct regulatory programs of the globin genes and their closely appended 3' neighbor, an odorant receptor gene. We conclude that both boundaries of the chicken  $\beta$ -globin domain are capable of playing functionally similar roles and that the same protein is a necessary component of the molecular mechanism through which these boundaries are defined.**

**Keywords:** chromatin domains/CTCF/globin genes/insulators/odorant receptors

## Introduction

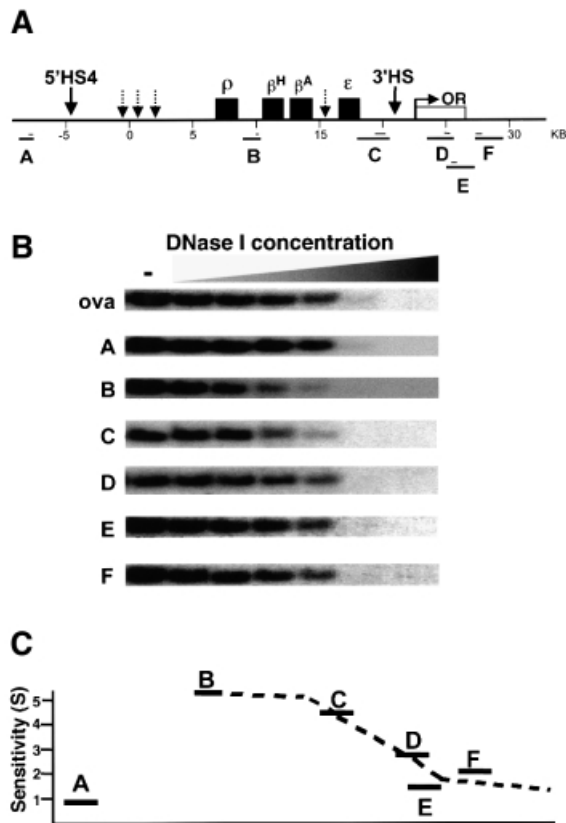
The formation of independent functional domains on eukaryotic chromosomes involves specific modifications of chromatin structure. Cytological studies of *Drosophila* polytene chromosomes distinguish adjacent heterochromatic and euchromatic regions (Bossy *et al.*, 1984; Rykowski *et al.*, 1988; Friedman *et al.*, 1991). At the molecular level, transcriptionally active genes and gene clusters are characterized by higher sensitivity to the action of nucleases (Weintraub and Groudine, 1976; Lawson *et al.*, 1982; Jantzen *et al.*, 1986; Farache *et al.*,

1990) and by higher levels of core histone acetylation relative to the surrounding 'inactive' chromatin (Hebbes *et al.*, 1988, 1994; O'Neill and Turner, 1995; Grunstein, 1997). The existence of active and inactive functionally distinct chromatin domains adjacent to each other raises the question of how they are kept distinct. It is known that certain DNA sequences, acting through the specific proteins bound to them, have properties that might make them suitable boundary elements. For example, the *scs* and *scs'* elements (reviewed in Bell and Felsenfeld, 1999) are localized at the proximal and distal ends of the 87A7 heat shock locus in *Drosophila*. They are capable of blocking activation of a reporter gene by an enhancer when placed between enhancer and promoter, and they also protect against position effects when they surround a reporter gene (Kellum and Schedl, 1991, 1992). The *gypsy* retrotransposon, also found in *Drosophila*, has similar enhancer-blocking and position effect suppressing activities.

In erythroid cells, the chicken  $\beta$ -globin genes are embedded in a 33 kb chromatin domain that is sensitive to DNase I and contains hyperacetylated histones (Hebbes *et al.*, 1988; Figure 1A). The 5' boundary of this domain has been determined precisely and is closely associated with a 'constitutive' DNase I hypersensitive site (HS) (5'HS4) present in every cell type and tissue that has been examined (Reitman and Felsenfeld, 1990; Hebbes *et al.*, 1994; Bell *et al.*, 1999). A DNA fragment containing this HS has the properties of an insulator: it blocks enhancer action directionally and it protects a reporter gene against position effects (Chung *et al.*, 1993, 1997; Pikaart *et al.*, 1998; Bell *et al.*, 1999; Recillas-Targa *et al.*, 1999). Furthermore, we have shown that the enhancer-blocking activity of this insulator requires the DNA-binding protein CTCF (Bell *et al.*, 1999).

These results are consistent with the idea that the 5'HS4 insulator may help to establish and maintain the 5' boundary of the globin domain. The need for such an element is further suggested by the presence, only 16 kb upstream of 5'HS4, of a folate receptor gene with a developmental program of expression that is distinct from that of the globin genes (Prioleau *et al.*, 1999). We have proposed that one role of the 5' boundary is to prevent cross-talk between the regulatory elements of these genes (Bell *et al.*, 1999; Prioleau *et al.*, 1999).

The existence of a 5' boundary element immediately raised questions about the location and properties of a 3' boundary. We have therefore examined in detail the 3' end of the chicken  $\beta$ -globin domain, and find striking structural similarities with the 5' end, although the two ends lack any long stretch of nucleotide sequence identity. A transition in the relative sensitivity of the chromatin to DNase I at the 3' boundary is marked by a constitutive HS. Its position maps precisely to a binding site for CTCF and



**Fig. 1.** A transition in DNase I sensitivity defines the 3' boundary of the chicken  $\beta$ -globin domain. The 3' boundary of generalized DNase I sensitivity is located between regions C and D. (A) Structure of the chicken  $\beta$ -globin locus is shown on the top. The four globin genes are indicated with black boxes. Selected HSs are shown by arrows. An open reading frame encoding the chicken odorant receptor protein (COR3 $\beta$ ) described in this study (Bulger *et al.*, 1999) is shown as a white box marked 'OR'. 5'HS4, the most upstream constitutive HS, marks the 5' chromatin boundary and functions as a chromatin insulator. Another constitutive HS, 3'HS, was identified in this study. Other erythroid-specific HSs are shown by arrows with dotted lines. Numbers immediately below the map are map units based on the arbitrary numbering system set (Villeponteau and Martinson, 1981). Restriction fragments A-F detected in DNase I sensitivity assays in (B) are shown below the map. Probes used are indicated as thin lines. A detailed description of DNA fragments A-F and probes is given in Materials and methods. (B) Generalized DNase I sensitivity of DNA fragments A-F visualized by Southern blot hybridization. Erythrocyte nuclei isolated from 11-day-old chick embryos were treated with increasing amounts of DNase I (from right to left lanes, 0, 0.2, 0.4, 0.6, 1.0, 2.0 and 5.0 U/ml). Restriction fragments A-F were detected by Southern blot hybridizations. Relative sensitivities to DNase I correspond to the extent of loss of signal intensities of each band. DNA fragments B and C are relatively sensitive to DNase I, while fragments D-F are resistant to DNase I. A DNA fragment derived from the ovalbumin gene, which is transcriptionally inactive in this cell type, and fragment A, which is located farther upstream of the 5' chromatin boundary, were used as DNase I resistant controls. (C) Quantitative representation of the DNase I sensitivities along the  $\beta$ -globin locus. Normalized DNase I sensitivity values (S) were calculated from the following equation (Pikaart *et al.*, 1992) and plotted on a graph:  $S = \log(G_D/G_U) / \log(O_D/O_U) \times T$ , where G and O are  $\beta$ -globin and ovalbumin band intensities for the undigested (U) or digested (D) samples and T is the size ratio of the ovalbumin to globin fragments. DNase I sensitivity drops significantly between C and D.

a DNA fragment containing this site has directional enhancer-blocking activity, which depends upon its ability to bind CTCF. Only 2 kb downstream of the 3' boundary

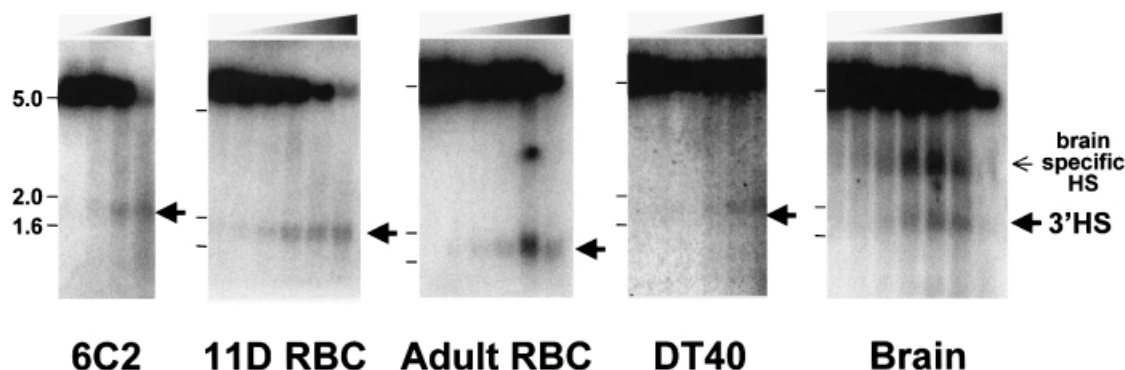
lies a gene that encodes an odorant receptor (OR) (Bulger *et al.*, 1999; this paper). We suggest that the enhancer-blocking activity of this boundary is a necessary component of the mechanism that maintains the distinct regulatory programs of these neighboring genes. Furthermore, the presence of a second element at the 3' end of the  $\beta$ -globin locus with such similarity to the one at the 5' end is strong evidence that both these elements are involved with the establishment or maintenance of chromatin domain boundaries.

## Results

### Chromatin sensitivity to DNase I defines the 3' chicken $\beta$ -globin domain boundary

In order to identify the 3' chromatin boundary of the chicken  $\beta$ -globin locus, we first surveyed the general DNase I sensitivity of a 14 kb region downstream of the embryonic  $\epsilon$ -globin gene (Figure 1A). In these experiments, nuclei isolated from the red blood cells (RBCs) of 11-day-old chick embryos were incubated with increasing concentrations of DNase I for a fixed amount of time. The rate of disappearance of individual restriction fragments with increasing DNase I concentration served as a measure of the accessibility of the chromatin in that region to the enzyme (Weintraub and Groudine, 1976; Lawson *et al.*, 1982; Jantzen *et al.*, 1986; Farache *et al.*, 1990). We found several considerations to be essential in accurate assessment of the relative sensitivities of these fragments. First, we carefully eliminated from our analysis any DNA fragments derived from regions containing sites that are hypersensitive to DNase I digestion (data not shown). Secondly, since the kinetics of digestion are dependent upon the length of the fragment examined, to avoid making large corrections for size, we chose restriction fragments of roughly equivalent length. A representative example of the data obtained by Southern blotting of such fragments is shown in Figure 1B. DNA fragments B and C are digested away with a lower concentration of DNase I relative to fragments D, E and F. When these results are quantified and normalized for length (see analysis in Figure 1 legend), a clear transition in the general DNase I sensitivity in this region is apparent (Figure 1C). The 3' end of fragment C is 4.8 kb downstream of the  $\epsilon$ -gene, while the 5' end of fragment D is 7.0 kb downstream of the  $\epsilon$ -gene. Thus, these results identify a 3' chromatin boundary located between 4.8 and 7.0 kb downstream of the  $\epsilon$ -gene. This position is consistent with the 3' boundary determined previously at lower resolution by both general DNase I sensitivity and histone acetylation pattern (Hebbes *et al.*, 1994).

A further examination of the DNase I sensitivity in the region defined above revealed an HS (not present in the fragments examined in Figure 1) that we designated as 3'HS. This site appears to be constitutive: it is present in all of the cell types we tested, including erythroid cells at early and late stages of erythropoiesis (6C2 cells, 11-day-old embryonic and adult RBCs) and non-erythroid cells such as DT40 and embryonic brain cells (Figure 2). No additional HSs were observed within a 10 kb region downstream of the 3'HS in RBCs from 11-day-old chick embryos.



**Fig. 2.** A constitutive hypersensitive site (3'HS) within the 3' chromatin boundary. Nuclei from various types of chicken cells were treated with increasing amounts of DNase I (from left to right lanes: 0, 0.06, 0.1, 0.2, 0.4 and 0.6 U/ml for RBCs and brain nuclei; 0, 8.0, 10, 20, 40 and 60 U/ml for DT40 and 6C2 cell nuclei). Genomic DNA was extracted and digested with *Kpn*I. In addition to a parental fragment, a hypersensitive site, 3'HS, was detected as marked by arrowheads, in all cell types tested. Cells tested are a chicken erythroid precursor derived cell line (6C2), erythroid cells from either 11-day-old embryo (11D RBC) or adult blood (Adult RBC), brain from 11-day-old embryos (Brain) and a lymphoma-cell derived cell line (DT40).

### **A DNA fragment including the 3'HS has enhancer-blocking activity**

We asked whether a fragment of DNA containing the 3'HS would have one of the functions of an insulator: the capacity to block an enhancer. To examine this, we made use of the same assay for enhancer-blocking activity previously employed to study the 5'HS4 insulator (Chung *et al.*, 1993, 1997; Bell *et al.*, 1999). In these assays, constructs carrying the neomycin resistance gene and an enhancer are stably transformed into the human erythroleukemia line K562; enhancer action is reflected in the number of neomycin-resistant colonies obtained in stably transfected K562 cells. Two DNA fragments spanning the 3'HS and one immediately adjacent to it were tested for enhancer-blocking activity in this assay (Figure 3A). Insertion of either of the fragments that contains the HS resulted in a reduction in colony number relative to controls. This reduction in colony number was roughly equivalent to that obtained with the 5' insulator and was not attributable to insert size as insertions of either a larger  $\lambda$  DNA fragment or a similarly sized fragment 3' to the boundary (3'HS-6) had no effect on colony number. The enhancer-blocking activities of the 3'HS and 3'HS-2 fragments were independent of the orientation in which these fragments were inserted (data not shown).

The enhancer-blocking activity of the 5' insulator is positional, i.e. to block an enhancer the insulator must be inserted between the enhancer and the promoter. As the results in Figure 3B show, in these assays, enhancer blocking by the 3'HS fragment was also positional: when the 3'HS fragment flanks the enhancer and promoter, it has no effect (Figure 3B, construct 5). Thus, the 3'HS blocks enhancers in a directional manner in agreement with the properties of an insulator.

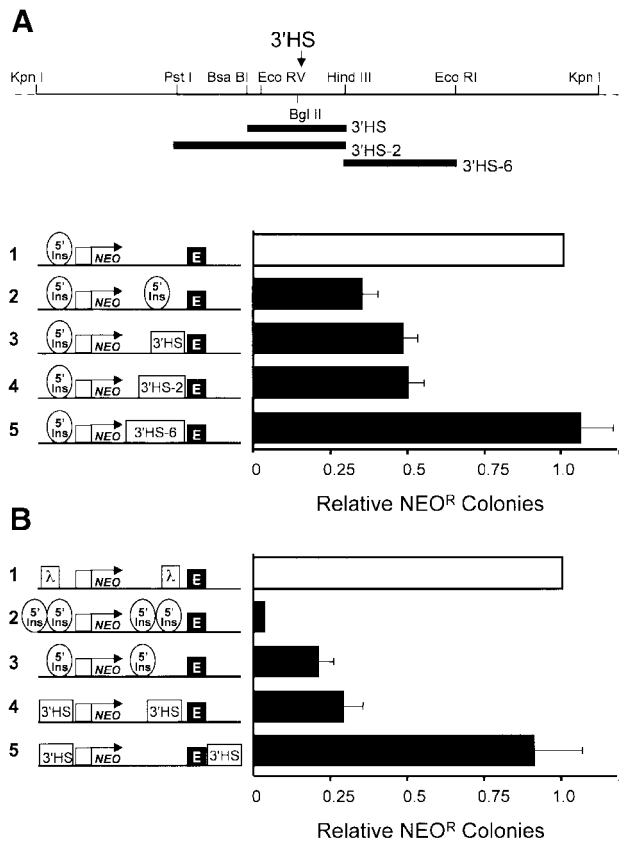
### **Identification of an adjacent gene**

In light of the enhancer-blocking properties of the 5' boundary, we have proposed that this element may play a necessary role in preventing cross-talk between neighboring genes (Bell *et al.*, 1999; Prioleau *et al.*, 1999). Given the enhancer-blocking activity of the 3' boundary, we searched for a gene at the 3' end of the globin locus. As

we reported in a recent paper (Bulger *et al.*, 1999), a gene encoding an OR lies only ~2 kb downstream of the globin 3'HS. This gene, COR3' $\beta$ , contains a complete open reading frame homologous to known OR proteins. Although most of the members of the OR protein family are expressed in the olfactory sensory epithelium, some are expressed in other tissues such as the cells migrating along the olfactory nerve and tissue adjacent to the neural tube (Nef and Nef, 1997), the taste buds (Abe *et al.*, 1993), developing heart (Drutel *et al.*, 1995), germ line (Parmentier *et al.*, 1992) and brain (Dreyer, 1998; Raming *et al.*, 1998). This pattern of expression is clearly different from that of the globin genes. Consistent with this difference, we discovered a brain-specific HS 0.9 kb downstream of the globin 3'HS (Figure 2). In addition, we were able to detect COR3' $\beta$ -specific poly(A)<sup>+</sup> transcripts in 10-day-old embryonic chick brain (data not shown).

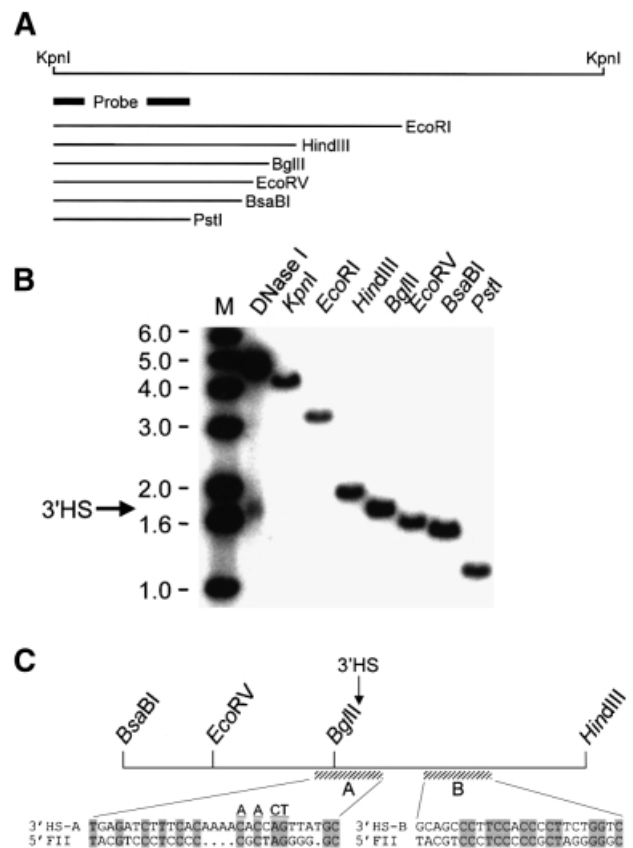
### **CTCF is required for the enhancer-blocking activity of the 3'HS**

To analyze the chromatin boundary further, we mapped the position of 3'HS. Because of the availability of restriction sites conveniently located near the 3'HS, we were able to map its position with accuracy (Figure 4). The location of the HS was nearly coincident with a *Bgl*III site. Although there were no striking similarities between the sequences surrounding the 5' and 3' HSs, we found two relatively weak homologies between the CTCF site in the 5' insulator (5'FII) and the 3'HS region (Figure 4C, 3'HS-A and B). The 3'HS maps directly to the 3'HS-A in this fragment (Figure 4). Although this alignment includes only a 12/30 identity (with a 4 bp insertion), a labeled 100 bp oligonucleotide containing 3'HS-A binds CTCF (Figure 5). With adult chicken RBC nuclear extracts, two slow mobility complexes were observed in a gel-shift assay with 3'HS-A (Figure 5A, lanes 1–9). The upper complex is competed by a 50-fold excess of cold 3'HS-A and 5'FII. Moreover, mutant forms of 5'FII competed to an extent that was directly proportional to their affinity for CTCF (Bell *et al.*, 1999). This complex had an identical mobility to that observed when 5'FII was used as a labeled probe (data not shown). Partially purified CTCF gave an



**Fig. 3.** Directional enhancer-blocking activity of the 3'HS. **(A)** The human erythroleukemic cell line K562 was stably transfected with the constructs shown on the left. Each construct has the neomycin resistance gene (NEO) driven by a human  $\beta^A$ -globin promoter with mouse  $\beta$ -globin HS2 as an enhancer. The DNA fragments 3'HS and 3'HS-2 include the DNase I HS 3'HS. 3'HS-6 does not contain the HS. For each construct, the 1.2 kb chromatin insulator fragment (5'Ins) including the 5'HS4 was placed upstream of the promoter in order to block influence from regulatory elements at the site of integration. The level of expression of each construct was measured as the number of neomycin-resistant colonies. Colony numbers obtained from construct 1, which does not have a DNA fragment between the promoter and the enhancer, were set at 100. Relative numbers of neomycin-resistant colonies are shown in the bar graph. We present the mean of five independent experiments. Enhancer-blocking activity resides in a DNA fragment containing the 3'HS. **(B)** Enhancer-blocking assays were performed using constructs shown to the left. In construct 1, a 2.3 kb fragment of  $\lambda$  DNA was inserted, as a spacer control, between the enhancer and the reporter. Thick bars show means of at least four independent experiments.

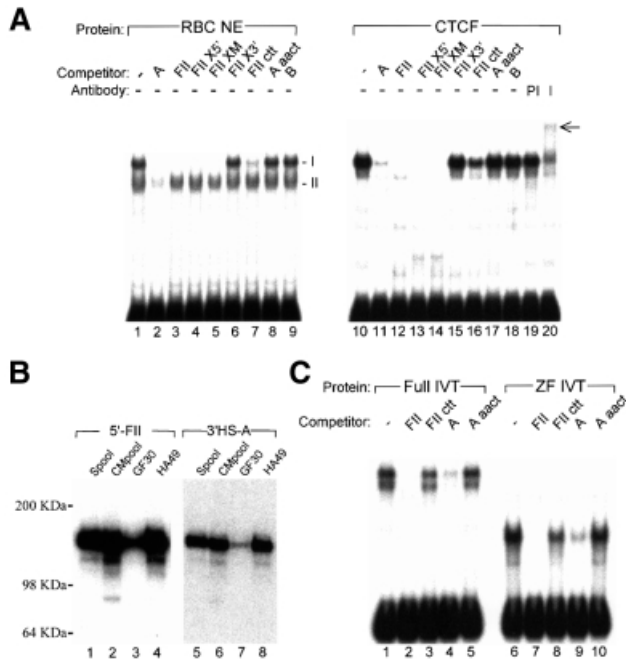
identical migration and competition profile, and this complex was specifically super-shifted with an antibody against CTCF (Figure 5A, lanes 10–20). Furthermore, in a Southwestern assay, both 5'FII and 3'HS-A bound specifically to a protein with an apparent mass of ~140 kDa that had been enriched through stepwise purification of 5'FII binding protein from adult chicken RBC nuclear extract (Figure 5B). We have demonstrated by peptide sequencing that the protein responsible for this Southwestern activity is CTCF (Bell *et al.*, 1999). Finally, the conclusion that 3'HS-A is a bona fide binding site for CTCF was confirmed by the observation that *in vitro* translated CTCF, and a peptide spanning its zinc finger domain, bound 3'HS-A with a specificity identical to that observed with native CTCF (Figure 5C).



**Fig. 4.** Sequences homologous to the 5' insulator element of the chicken  $\beta$ -globin locus are found at the site of the 3'HS. The position of the 3'HS was measured by the indirect end-labeling method and the strategy is shown in **(A)**. Nuclei from 11-day-old chick embryos were treated with 0.4 U/ml DNase I, from which genomic DNA was extracted and digested with *KpnI*. In **(B)**, the position of the 3'HS (arrow) was compared with the migration of genomic fragments of known length. The 3'HS hypersensitive fragment co-migrates with a fragment derived from *BglII* digestion. **(C)** Sequences homologous to FII from 5'HS4 (Chung *et al.*, 1997; Bell *et al.*, 1999) are found at or close to the sites of 3'HS, 3'HS-A and 3'HS-B, respectively. Alignment of the sequences 3'HS-A and 3'HS-B with the sequences of the 5'FII is shown. Conserved bases are shaded. Bases altered to generate a mutant site are underlined.

Within 5'FII, base pairs at the 3' end of the fragment are essential for CTCF binding (Bell *et al.*, 1999). In that site, a 12 bp transversion at the 3' end was sufficient to ablate CTCF binding *in vitro* and enhancer blocking *in vivo* completely (FIIX3' in Figure 5). Moreover, nearly all of the binding to CTCF was eliminated by a more minimal 3 bp transversion in this same region of 5'FII (FII ctt in Figure 5). In the context of the 3'HS-A site, we substituted each of the base pairs that is identical to the 3' region of the 5'FII site with the corresponding base transversions (A aact in Figure 5). These mutations of 3'HS-A were sufficient to eliminate CTCF binding. Although 5'FII has a more uniform similarity to 3'HS-B than to 3'HS-A, 3'HS-B showed no detectable CTCF binding (Figure 5A).

We have shown that a fragment containing the 3'HS has the enhancer-blocking properties of an insulator (Figure 3). Deletion of 3'HS-A from this fragment resulted in an essentially complete loss in enhancer-blocking activity (Figure 6). Furthermore, the 3'HS-A site was independ-

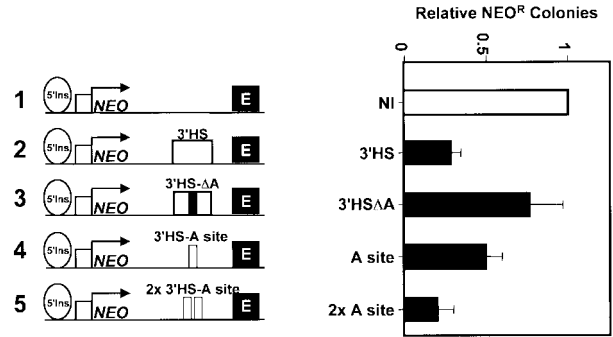


**Fig. 5.** Sequences derived from the 3'HS are bound specifically by CTCF. **(A)** Gel retardation analysis of complexes formed with the 3'HS-A sequence. Duplex 100mer oligonucleotides containing the 3'HS-A site were incubated with adult chicken RBC nuclear extracts (lanes 1-9) or partially purified CTCF (lanes 10-20; Bell *et al.*, 1999). Two complexes observed following incubation with nuclear extracts are labeled I and II. Complexes were competed with 50-fold excesses of unlabeled competitor oligonucleotides containing either the 3'HS-A (lanes 2 and 11), 5'FII (lanes 3 and 12), FII X5'-mutant (lanes 4 and 13), FII X5M-mutant (lanes 5 and 14), FII X3'-mutant (lanes 6 and 15), FII ctt-mutant (lanes 7 and 16; Bell *et al.*, 1999), 3'HS-A aact-mutant (lanes 8 and 17) or 3'HS-B (lanes 9 and 18) sites. Pre-immune antibodies (lane 19) or anti-CTCF antibodies (lane 20) were added to the binding reactions, and supershifted material is indicated by the arrow at the right. **(B)** Southwestern analysis of oligonucleotide duplexes binding to immobilized fractions of purified CTCF. Fractions of increased CTCF purity were separated by polyacrylamide gel electrophoresis and immobilized on PVDF membrane. The positions of molecular weight markers are indicated. A single gel blot was cut in two and probed with <sup>32</sup>P-labeled oligonucleotide duplexes containing the 5'FII (lanes 1-4) and 3'HS-A (lanes 5-8) sites. Following washing, probed blots were exposed to autoradiographic film for 3 min and 2 h, respectively. **(C)** Gel retardation analysis of complexes formed between the 3'HS sequence and *in vitro* translated CTCF proteins. Duplex 100mer oligonucleotides containing the 3'HS-A site were incubated with *in vitro* translated proteins representing the full-length (lanes 1-5) or the zinc finger domain (lanes 6-10) of CTCF. Complexes were competed with 50-fold excesses of unlabeled competitor as indicated.

ently capable of blocking an enhancer in our assay (Figure 6).

## Discussion

In this study, we analyzed the 3' chromatin boundary of the chicken  $\beta$ -globin locus and found striking similarities with the 5' chromatin boundary. Previous attempts to locate the 3' boundary have provided ambiguous results. One study suggested that the 3' chromatin boundary is located between 5 and 9 kb downstream of the  $\epsilon$ -globin gene (Hebbes *et al.*, 1994), another concluded that it lies at least 7 kb downstream of the  $\epsilon$ -globin gene (Stalder *et al.*, 1980), and a third located the 3' chromatin boundary much



**Fig. 6.** The CTCF binding site found in the 3'HS is sufficient and necessary for enhancer-blocking activity. The enhancer-blocking activities of the 3'HS-A variants indicated were measured in the colony assay. In construct 3, a 56 bp fragment spanning the A-site has been deleted from the 400 bp 3'HS fragment and inserted into construct 1. One (construct 4) and two (construct 5) copies of the 3'HS-A site alone confer enhancer-blocking activity.

further downstream (Verreault and Thomas, 1993). Some of these earlier experiments tested the general DNase I sensitivity of DNA fragments now known to contain DNase I HSs. The presence of such sites results in rapid loss of target restriction fragments in the assay, which can be mistaken for general nuclease sensitivity over the entire region. We show here that a transition in general DNase I sensitivity occurs in the region from 4.8 to 7.0 kb downstream of the embryonic  $\beta$ -globin gene.

We found a constitutive HS at the 3' end of the domain similar to what is observed at the 5' boundary. Further downstream we recently identified a complete open reading frame encoding an OR protein (COR3 $\beta$ ; Bulger *et al.*, 1999) and there is another putative OR gene still further downstream (Staines and Thomas, 1999). Here we also show (Figure 2) that there is a brain-specific HS just upstream of COR3 $\beta$ . The constitutive 3'HS lies between the globin genes and COR3 $\beta$ . Since this was reminiscent of the properties and location of the 5'  $\beta$ -globin insulator element, we analyzed the 3' boundary region for insulator activity. We found that a DNA fragment containing the 3'HS was able to block enhancer activity in a position-dependent manner in stably transfected K562 cells. Sequence comparisons between this HS and that of the 5' insulator element (5'HS4) revealed, at the position of the 3'HS, a sequence of moderate similarity. A direct comparison of their DNA binding activities in gel-shift experiments showed that these sequences bind the same protein, CTCF.

### Comparison of the 3' and 5' boundary elements

As noted above, the DNA sequences around the 5' and 3' boundaries of the  $\beta$ -globin locus share many properties. Most significant is the ability of both to block enhancer action in a directional manner. An equally important and related property is the ability to bind *in vitro* the same protein, CTCF. We have recently shown (Bell *et al.*, 1999) that the ability to bind CTCF is necessary and sufficient to confer enhancer-blocking activity on region 5'FII of the  $\beta$ -globin 5'HS4. CTCF is an 11 zinc finger protein that has been implicated in both activation (Klenova *et al.*, 1993; Vostrov and Quitschke, 1997) and repression of expression in other systems (Arnold *et al.*, 1996). Moreover, binding sites for CTCF are found in other reported

enhancer-blocking elements associated with insulator function at non-erythroid loci (Bell *et al.*, 1999).

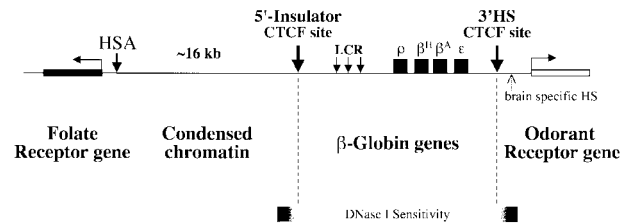
Wide sequence variation among CTCF binding sites has been noted in other cases (Burcin *et al.*, 1997). Although the 3' and 5' sites both bind CTCF, their affinities are different (Figure 5). Nonetheless, the enhancer-blocking activities of the two sites are similar (Figure 3). CTCF has been shown to use different subsets of fingers at different binding sites (Filippova *et al.*, 1996). This may explain the relatively weak homology between 5'FII and the 3' site shown in Figure 4C. For the same reason, CTCF might engage essential cofactors differently at the two sites, which could affect the strength of enhancer blocking.

There are some differences between the 3' sequence element we describe here and the 1.2 kb DNA fragment containing 5'HS4: the 5' element contains additional distinct sequences that protect against position effects (F.Recillas-Targa, M.J.Pikaart, B.Burgess-Beusse, A.C. Bell and G.Felsenfeld, unpublished). The 400 bp fragment containing the 3'HS that we describe here does not protect against position effects (data not shown). It may be that the requirements for an effective 3' boundary element are somewhat different from those for the 5' element, since at the 5' end the folate receptor gene is separated from the globin locus by ~16 kb of condensed chromatin. Furthermore, the 3' condensed chromatin region must certainly be disrupted in those olfactory cells where the OR gene is expressed. In contrast, the 5' condensed region contains no coding sequences and is probably never in an active chromatin conformation.

#### **Biological significance of enhancer-blocking activity**

The position of 5'HS4 coincides with the 5' boundary between the active and inactive chromatin domains of the  $\beta$ -globin locus (Chung *et al.*, 1993; Hebbes *et al.*, 1994). It has seemed reasonable to speculate that 5'HS4 in some way determines the boundary, and that enhancer-blocking activity plays a role in its establishment. The results presented here show that both ends of the  $\beta$ -globin locus possess such elements, and thus provide a persuasive argument that these are indeed functional elements in the establishment and/or maintenance of boundaries. We note that in the case of the *scs/scs'* elements of *Drosophila*, the proteins binding to the boundaries are quite distinct (Zhao *et al.*, 1995; Gaszner *et al.*, 1999). It appears that in contrast the two globin boundaries have at least one common binding component, although as noted above other components may differ.

We have speculated that the 5' boundary serves to limit the influence of folate receptor-specific and globin-specific regulatory elements to their respective loci (Prioleau *et al.*, 1999). Just as in the case of the 5' boundary, which lies between the  $\beta$ -globin locus and an upstream folate receptor gene with a different developmental pattern of expression, the 3' boundary lies between the nearby strong regulatory elements of the  $\beta$ -globin locus and the putative promoter for an OR gene, each with a distinct program of tissue-specific expression (Figure 7). The 3'HS may perform similar functions, preventing cross-talk between the downstream regulatory elements in the  $\beta$ -globin locus (the strong  $\beta/\epsilon$  enhancer and the  $\epsilon$  promoter) and the OR regulatory elements, which we suggest may be located at



**Fig. 7.** Summary of the chromatin structure at the boundaries of the chicken  $\beta$ -globin locus in erythroid cells. A schematic view of the region extending from the chicken  $\beta$ -globin locus is shown. CTCF binds to both the 5' and 3' chromatin boundaries (Bell *et al.*, 1999; this study), and may be involved in shielding the  $\beta$ -globin locus from regulatory effects from neighboring genes: the upstream folate receptor gene (Prioleau *et al.*, 1999) and the downstream odorant receptor gene (Bulger *et al.*, 1999; this study), respectively.

the brain-specific HS 0.9 kb downstream of the globin 3'HS (~1.1 kb upstream of the coding sequence of COR3 $\beta$ ; Figure 2). Tissue-specific HSs often mark enhancer or promoter elements responsible for the tissue specificity (Reitman and Felsenfeld, 1990). The existence of the brain-specific HS therefore suggests that COR3 $\beta$  is active in brain cells. Our observation of a COR3 $\beta$  transcript in embryonic brain tissue (data not shown) is direct evidence for such activity. Consistent with this observation, transcripts of the mouse homologs MOR3 $\beta$ 1 and MOR3 $\beta$ 2 have been observed in olfactory epithelium (Bulger *et al.*, 1999).

The observed insensitivity to DNase I (Figure 1) over COR3 $\beta$  in erythroid cells indicates that at least in most of these cells the chromatin of this OR gene is in an inactive condensed conformation. This is confirmed by the observation that histone acetylation levels are low over the COR3 $\beta$  promoter region (M.Litt and G.Felsenfeld, unpublished observations). RT-PCR measurements (data not shown) do detect a low level of RNA from this gene in 5- and 10-day-old erythroid cells, suggesting that at least in a few cells of the population the gene might be in an active conformation. We note that low levels of transcripts have been detected from at least one OR gene (HPFH1OR) near the human  $\beta$ -globin locus and two (MOR3 $\beta$ 2 and 3) near the mouse locus in some, but not all erythroid cells (Feingold *et al.*, 1999).

The identification of the 3' boundary element is consistent with tentative reports of enhancer-blocking activity in other globin gene systems. Recent studies in transgenic mice of the human  $\beta$ -globin locus (Tanimoto *et al.*, 1999) show that inversion of the locus control region (LCR) results in greatly reduced expression of all  $\beta$ -like globin genes. One possible explanation of this result is that the inversion places 5'HS5 of the LCR between the other LCR elements and the globin locus. It has been reported (Chung *et al.*, 1993; Li and Stamatoyannopoulos, 1994) that 5'HS5 has enhancer-blocking activity. In support of this view is the further observation (Tanimoto *et al.*, 1999) that a tagged  $\epsilon$ -globin gene placed upstream of HS5 in a normally oriented LCR is not expressed, whereas one placed immediately downstream of the LCR is active.

The mechanism by which boundaries are established is still not clear. It has been suggested (Pikaart *et al.*, 1998; Bell and Felsenfeld, 1999) that the suppression of position effects observed with the 5'  $\beta$ -globin insulating element may reflect either an ability to protect the reporter from the



action of histone deacetylases, or an ability to sequester histone acetylases within the protected region. Either of these actions would help to preserve the transcriptionally active status of the domain against outside influences from nearby endogenous regulatory elements or condensed chromatin. The structure of such a domain remains to be investigated. Among other possibilities, the proteins bound at the 3' and 5' boundaries may interact with each other or with the nuclear architecture to create physically defined domains. Now that the location and identity of the boundaries are known, this proposition can be tested.

## Materials and methods

### DNase I sensitive assay

**Preparation of nuclei.** Embryonated White Leghorn chicken eggs (11-day-old) and adult chicken blood were obtained from Truslow Farms, Chestertown, MD. Erythrocytes were harvested from a dozen eggs, collected in phosphate-buffered saline (PBS) containing 5 mM EDTA and washed in 50 ml of buffer A [10 mM Tris-HCl pH 7.5, 80 mM NaCl, 6 mM MgCl<sub>2</sub>, 0.2 M phenylmethylsulfonyl fluoride (PMSF) and 10 µg/ml aprotinin]. Cells were lysed in buffer A with 0.1% Triton X-100. Nuclei were pelleted, washed in the same buffer three times, and resuspended in RSB buffer (10 mM Tris-HCl pH 7.5, 10 mM NaCl, 3 mM MgCl<sub>2</sub>) at a concentration of 20 A<sub>260</sub>. Nuclei from 6C2 cells and DT40 cells were prepared following the same protocol. Nuclei were digested with varying concentrations of DNase I (Worthington), typically 0–5 U/ml for 10 min. DNase I-treated DNA from chicken brain nuclei was kindly provided by Dr M.-N. Prioleau. Each DNA sample (10 µg) was digested overnight with restriction enzymes and 8 µg of DNA/lane were electrophoresed through a 0.8% agarose gel. Southern hybridizations were performed at 65°C using QuickHyb (Stratagene). After a final wash in 0.2× SSC–0.1% SDS at 65°C, filters were either autoradiographed or exposed to a Molecular Dynamics PhosphorImager to measure intensities of hybridized signals.

**Hybridization probes.** DNA fragments in Figure 1 were detected as follows: A, a 1.4 kb *Bam*HI fragment detected with probe P1 (450 bp; *Nco*I–*Bam*HI at –7.9 to –7.5 map units); B, a 2.1 kb *Bam*HI–*Hind*III fragment detected with probe i116 (450 bp; *Pst*I–*Bam*HI at 9.5–9.9 map units); C, a 2.4 kb *Bgl*II–*Bsa*BI fragment detected with probe i112 (1000 bp; *Kpn*I–*Pst*I at 23.3–24.3 map units); D, a 2.3 kb *Bgl*II–*Bsa*BI fragment detected with probe P9 (500 bp; *Bam*HI–*Eco*RI at 28.1–28.6 map units); E, a 3.1 kb *Bam*HI–*Bam*HI fragment detected with probe P9; F, a 3.2 kb *Bam*HI–*Bam*HI fragment detected with probe i87 (500 bp; *Nco*I–*Nco*I at 31.6–32.1 map units); Ova, a 1.4 kb *Bam*HI–*Hind*III fragment detected with a probe i104 (500 bp; *Eco*RI–*Sma*I derived from a clone OVI.8; Woo *et al.*, 1981), provided by Dr Marc Reitman. The DNase I HS, 3'HS, was detected by digestion of nuclei with increasing amounts of DNase I as above followed by digestion of extracted DNA with *Kpn*I and probing with i112 (1000 bp; *Kpn*I–*Pst*I at 23.3–24.3 map units).

### Plasmid constructions

Constructs used in Figure 3A: construct 1 was pNI (Chung *et al.*, 1997) and construct 2 was pJC5-4 (Chung *et al.*, 1993). In order to create constructs 3 (pJC3'HS), 4 (pJC3'HS-2) and 5 (pJC3'HS-6), DNA fragments '3'HS', '3'HS-2' and '3'HS-6' as indicated in Figure 3 were PCR amplified from cosmid +ENH (Barton *et al.*, 1990). Primers used were ns11 (5'-AGGCGCGCCAGATTTTCATCACCTTCTCCCA-TGC-3') and ns12 (5'-AGGCGCGCCAAGCTTAGCTATGTGCCCA-AAAGGATGAGGA-3') for '3'HS', ns12 and ns14 (5'-AGGCGCGC-CCTTCCTGTGAAAACATCACCTAGCCA-3') for '3'HS-2', and ns13 (5'-AGGCGCGCCTTCCTGTGAAAACATCACCTAGCCAT-3') and ns20 (5'-AGGCGCGCCGGGTTGGGCTGAGTGGGCTT-3') for '3'HS-6'. Each amplified fragment was inserted in the *Asc*I site of pNI.

Constructs used in Figure 3B: the constructs 1, 2 and 3 were pJC3-4, pJC13-1 and pJC5-4 (Chung *et al.*, 1993), respectively. Construct 4 (pJC3'double) was derived from pJC3'HS (see above). The 1.2 kb of 5'Ins sequence at *Xba*I site was removed and replaced with the PCR-amplified 3'HS DNA fragment. The construct 5 (3'pJC3') was derived from the plasmid pJC3'double. '3'HS' at the *Asc*I site was removed, and inserted at the *Nde*I site.

Constructs used in Figure 6: the constructs 1 and 2 were pNI (Chung *et al.*, 1997) and pJC3'HS (see above). Constructs 3–7 were derived from pNI. 3'HS-ΔA was generated by two-step overlapping PCR using the cosmid +ENH (Barton *et al.*, 1990) as the first template and two sets of primers ns11 and ns77 (5'-TTCAGAGGCATAGCAGACGCAGTT-CTCCGAGTCTGACTTT), and ns12 and ns76 (5'-AAAGTCA-GACTCGGAGAAGTGCCTGTGCTATGCCTCTGAA). The product of that reaction was used as the template for the second round of PCR using primers ns11 and ns12. 3'HS-A and 2×~3'HS-A were created by inserting, in one and two copies, respectively, duplexed oligomers identical to those used in DNA-binding assays into the *Asc*I site of pNI.

### Colony assay by stable transfection of K562 cells

Colony assays were performed as described previously (Chung *et al.*, 1993). Briefly, exponentially growing K562 cells were harvested, washed in PBS, and resuspended in PBS at 2 × 10<sup>7</sup> cells/ml. Linearized plasmid (250 ng) was transfected by electroporation with a Bio-Rad Gene Pulser at 200 V and 960 µF. The cells were transferred to 20 ml of growth medium and cultured for 48 h. For drug selection, 3 ml of the cell suspension were mixed with 27 ml of growth media complemented with 0.3% cell culture agar and 750 µg/ml active G418 (Gibco). Colonies grown in the presence of G418 were counted 2–3 weeks after selection.

### DNA sequencing and analysis

Plasmids used as sequencing templates were p556 and p932, originally derived from the cosmid p918, kindly provided by Dr Marc Reitman (Barton *et al.*, 1990). In this study, nucleotide sequences were determined from 21.0 to 28.2 map units of the chicken β-globin locus (DDBJ/EMBL/GenBank accession No. L17432).

### Gel mobility shift assays

Oligonucleotides used as templates and competitors were synthesized with an Applied Biosystems Synthesizer, and purified on urea-polyacrylamide gels prior to use (Sambrook *et al.*, 1989). Each upper strand was labeled at the 5' end with polynucleotide kinase (New England Biolab) and [γ-<sup>32</sup>P]ATP. The reaction was terminated by adding EDTA at 10 mM and heating at 90°C for 10 min. To prepare duplex DNA, 1.5× molar excess of the corresponding lower strand was added, heated at 94°C for 10 min, followed by slow cooling to room temperature. To remove salt and unincorporated nucleotides, annealed products were spun through a Sephadex G50 spin column (Pharmacia). To prepare cold competitors, strands were annealed in TEN buffer (10 mM Tris-HCl pH 7.9, 1 mM EDTA, 50 mM NaCl) by heating at 94°C for 10 min, and then slowly cooling to room temperature. *In vitro* translated CTCF, nuclear extract and partially purified CTCF were prepared as previously described (Bell *et al.*, 1999). For gel mobility shift assays, 20 fmol of radiolabeled probe, ~1 µl of nuclear extract (3 µg/µl) or S-pool fraction (~1 µg/µl), 1 µg of poly dG-dC, 1 mM dithiothreitol (DTT), 0.5% Triton X-100 and 1 pmol of competitor oligonucleotide, where indicated, were mixed in 1× GS buffer (20 mM HEPES pH 7.9, 150 mM KCl, 5 mM MgCl<sub>2</sub>, 5% glycerol) at room temperature for 15 min. Samples were electrophoresed on 5% acrylamide gels in 0.5× TBE (45 mM Tris-borate pH 7.5, 1 mM EDTA) at room temperature. Southwestern blotting was performed as previously described (Bell *et al.*, 1999).

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