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The Protein CTCF Is Required for the Enhancer Blocking Activity of Vertebrate Insulators

Adam C. Bell, Adam G. West, and Gary Felsenfeld*
Laboratory of Molecular Biology
National Institute of Diabetes and
Digestive and Kidney Diseases
National Institutes of Health
Bethesda, Maryland 20892-0540

Summary

An insulator is a DNA sequence that can act as a barrier to the influences of neighboring *cis*-acting elements, preventing gene activation, for example, when located between an enhancer and a promoter. We have identified a 42 bp fragment of the chicken β-globin insulator that is both necessary and sufficient for enhancer blocking activity in human cells. We show that this sequence is the binding site for CTCF, a previously identified eleven–zinc finger DNA-binding protein that is highly conserved in vertebrates. CTCF sites are present in all of the vertebrate enhancer-blocking elements we have examined. We suggest that directional enhancer blocking by CTCF is a conserved component of gene regulation in vertebrates.

Introduction

Enhancer-mediated activation is a fundamental mechanism of gene regulation in eukaryotes. Enhancers can act over large distances to activate transcription, independent of their orientation and position relative to the promoter. In many cases, if given access, enhancers can act promiscuously to activate transcription of heterologous promoters. Genome sequencing has revealed many cases where differentially regulated genes neighbor each other at distances over which enhancers might act, yet the genes are independently regulated. Taken together, these facts suggest the need for mechanisms that prevent the inappropriate action of an enhancer on a neighboring locus (see for example Hagstrom et al., 1996 and Zhou et al., 1996). This restriction must be achieved, at least in some cases, without impeding the action of the enhancer within its native locus. A DNA element able to function in this way would in effect constitute a boundary to the action of an enhancer, preventing it from acting across the boundary, while otherwise leaving the enhancer unimpeded. This property is one of the defining characteristics of a kind of regulatory element only recently recognized: the insulator (Kellum and Elgin, 1998; Bell and Felsenfeld, 1999; Udvardy, 1999).

The first DNA sequences to be described as having the properties of an "insulator" were the *scs* and *scs′* elements of *Drosophila*, initially identified as marking the chromatin boundaries of a heat shock locus (Udvardy et al., 1985). When *scs* elements are placed on either side of a gene for eye color and introduced into *Drosophila*, the resulting flies all have similar eye color independent of the transgene's site of integration, an indication that *scs* has protected the reporter gene from both negative and positive endogenous influences, or "position effects" (Kellum and Schedl, 1991, 1992). Another *Drosophila* insulator element, *gypsy*, was first identified because of its ability to block the action of an enhancer on a promoter when it lies between them, but not otherwise (Holdridge and Dorsett, 1991; Geyer and Corces, 1992; Dorsett, 1993). Studies of these elements have led to a working definition of insulators: they protect against position effects and/or they block enhancer action in a directional manner. In the cases of both *scs′* and *gypsy*, proteins have been identified that bind specifically to the DNA elements and are, at least in part, responsible for mediating insulator activity (Geyer and Corces, 1992; Zhao et al., 1995).

Insulator elements have also been identified in vertebrates (Chung et al., 1993, 1997; Robinett et al., 1997; Zhong and Krangel, 1997), but the protein(s) responsible for their activity has been elusive. In earlier work from our laboratory, we described a 1.2 kb DNA element with strong enhancer blocking activity, which was derived from the 5′ end of the chicken β-*globin* locus (Chung et al., 1993, 1997). This region contains a DNase I–hypersensitive site, present in all tissues (Reitman and Felsenfeld, 1990), and its position coincides almost exactly with the region of transition between an active chromatin conformation, marked both by histone hyperacetylation and heightened sensitivity to DNase I, and an inactive domain extending farther 5′ that is insensitive to nuclease and less highly acetylated (Hebbes et al., 1994).

Recently we reported that we were able to reduce this element to a 250 bp "core" fragment that accounted for a significant portion of the globin insulator enhancer blocking activity (Chung et al., 1997). We describe here the identification of a single DNA-binding site within the core that is necessary and sufficient for enhancer blocking. We have purified a protein that binds to this site and show that the affinity of various mutant binding sites for this protein is directly proportional to their capacity to act as insulators in vivo. The DNA-binding protein responsible for this activity is CTCF (CCCTC-binding factor), a highly conserved and ubiquitous DNA-binding protein implicated in both transcriptional silencing and activation (Baniahmad et al., 1990; Lobanenkov et al., 1990; Klenova et al., 1993; Filippova et al., 1996; Burcin et al., 1997; Vostrov and Quitschke, 1997).

We find that functional CTCF-binding sites are also present in other insulators from diverse vertebrate species. Although these elements derive from a variety of genetic loci, they are all located between independently regulated genes. We suggest that directional enhancer blocking by CTCF is a conserved functional component of vertebrate domain boundaries.

*To whom correspondence should be addressed (e-mail: gxf@vger.niddk.nih.gov).

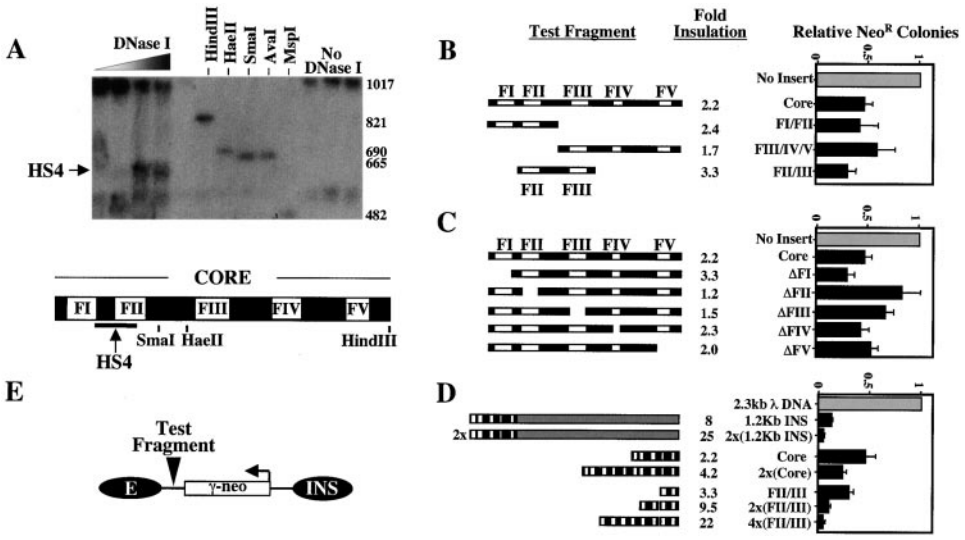


Figure 1. Fine Mapping of the Insulator Core

(A) The position of HS4 was measured by comparing the migration of the fragment generated by limited digestion of chicken erythrocyte chromatin with DNase I with the migration of genomic DNAs of known length. The position of HS4 relative to previously defined DNase I footprints (Chung et al., 1997) is indicated. (B-D) Results of enhancer blocking assays in which the elements indicated were placed between enhancer and promoter as shown in (E). A schematic of each inserted element is shown, as well as the relative numbers of colonies observed, and the numerical value of the insulation effect (fold insulation) relative to the uninsulated controls (No Insert [pNI] and λ DNA [pJC3-4]). The data presented in this figure represent the average of at least 4 independent assays. (B) Enhancer blocking activity of fragments of the core. (C) Effect on enhancer blocking of deletion of footprinted regions from the core. (D) Increasing enhancer blocking observed when insulating elements were multimerized. (E) A schematic diagram of the construction used to test various DNA fragments for enhancer blocking activity.

Results

We showed in earlier studies that a 1.2 kb DNA element at the 5′ end of the chicken β-*globin* locus, corresponding to a constitutive DNase I–hypersensitive site (5′HS4), functions as an insulator in an enhancer blocking assay. This assay tests the ability of a sequence to prevent activation of a gene for neomycin resistance by a strong enhancer when the construct is stably transformed into an erythroleukemia cell line (Chung et al., 1993). The insulator effect is manifested by a marked reduction in the number of colonies resistant to G418 only when the globin insulator is placed between enhancer and promoter. By this same assay, we showed that a large part of the insulator activity is contained in a 250 bp GC-rich "core" fragment at the 5′ end of the 1.2 kb element (Chung et al., 1997). HS4 maps precisely within this core region, consistent with its significance in vivo (Figure 1A).

Fine Mapping of Directional Enhancer-Blocking Sequences

DNase I footprinting of the core revealed five protected regions (FI to FV, illustrated in Figure 1A; Chung et al., 1997). We chose to focus on this fragment to identify an insulator protein–binding site. We divided the core into separate segments and carried out enhancer blocking assays with each fragment. Splitting the core between FII and FIII generated two fragments (FI/FII and FIII/IV/V) each of which had some enhancer blocking activity. However, a fragment containing only FII and FIII had greater activity than the entire core (FII/III, Figure 1B).

Deletion analyses confirmed that FII and FIII were responsible for the majority of the enhancer blocking activity of the core. While deletion of FI slightly increased the enhancer blocking effect, deletion of FII and FIII significantly reduced enhancer blocking activity (Figure 1C). Deletions of FIV and FV were essentially neutral. Insertion of an increasing number of copies of the FII/III fragment between the enhancer and the promoter resulted in a stepwise increase in blocking activity; an increase was also observed for the 1.2 kb insulator and the core (Figure 1D). Taken together these results show that the FII/III fragment is the functional enhancer-blocking region of the core.

Further analysis of the FII/FIII fragment showed that removal of the "spacer" sequence between FII and FIII resulted in even stronger blocking activity (Figure 2A). In fact, by removing sequences adjacent to FII, we obtained a 42 bp sequence spanning FII that alone possessed a blocking activity nearly equal to that of the full 1.2 kb insulator. Consistent with its functional importance, we note that the position of FII is coincident with that of HS4 in nuclei (Figure 1A). Importantly, enhancer blocking by FII displays the same position-dependence as that observed for the full 1.2 kb insulator (Figure 2B; Chung et al., 1997). When placed either upstream of the enhancer or downstream of the promoter, FII has essentially no effect on colony number in our assay. Thus, in order to affect expression, FII must be located between the enhancer and the promoter.

Identification of a Candidate Enhancer-Blocking Protein

Since the FII fragment had the strongest activity, we focused our attention on identifying proteins that bound

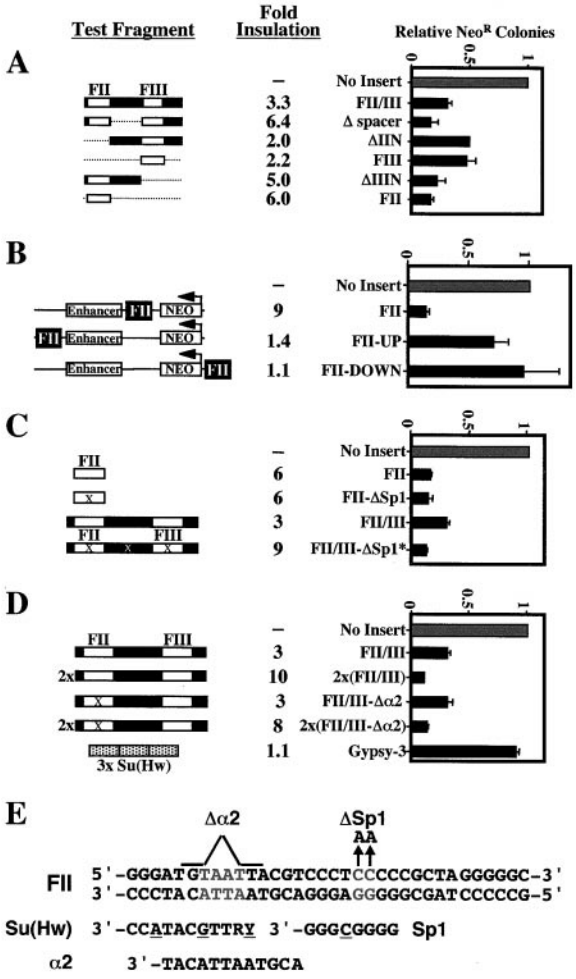


Figure 2. Identification of a Minimal Enhancer-Blocking Site

(A) A 90 bp fragment spanning FII and FIII was subjected to further deletion. Deleted regions are indicated by a dashed line. The effects of these deletions on enhancer blocking are shown. (B) Examination of the effect of the relative positions of enhancer and promoter on the enhancer blocking effect of FII in the colony assay. (C) Enhancer blocking activities of mutants of FII or FII/III aimed at assessing the contribution of binding sites for Sp1 or (D) α2 and Su(Hw) binding site homologies. (E) Alignments of Sp1, α2, and Su(Hw) binding sites with FII. The grayed base pairs TAAT were deleted to examine the contribution of the α2 and Su(Hw) homologies to enhancer blocking and DNA binding; the grayed CC→AA mutation was used to reduce Sp1 binding to FII and FII/III (Anderson and Freytag, 1991). The enhancer blocking data presented in this figure represent the average of 2–5 independent assays for each construction.

to it. A comparison of the sequence of FII with that of known transcription factor–binding sites revealed several potentially significant homologies. An Sp1 consensus sequence lies in the middle of the fragment and a sequence homologous to a yeast α2-binding site (Sauer et al., 1988) overlaps a partial match to the binding site of the *Drosophila* protein suppressor of Hairy-wing (Su[Hw]) (Figure 2E; Geyer and Corces, 1992). To test whether any of these homologies could account for the blocking activity of FII, we introduced mutations that were predicted to reduce dramatically the affinity of each of these proteins for this sequence. A deletion of 4 base pairs within the region that overlaps both the α2- and the Su[Hw]-binding sites had no effect on the blocking activity of the FII/III fragment (Figure 2D). Furthermore, a 100 bp fragment, derived from the *Drosophila gypsy* element, that contains three canonical Su[Hw]-binding sites, had no activity in our assay. We conclude that neither the Su[Hw] nor the α2 site can account for the activity of FII.

Likewise, in the context of FII, mutation of the Sp1 consensus had no effect on blocking activity; in fact, mutation of each of the three potential Sp1-binding sites in FII/III resulted in substantially increased activity (Figure 2C). Sp1 may act as an inhibitor of enhancer blocking in our assay. This may also explain the inhibitory effect of the "spacer" sequence between FII and FIII noted above.

To determine which sequences within FII are responsible for its activity we made multiple transversions (C→A and G→T) across the 5′, middle (M), and 3′ regions of the fragment (Figure 3A). All of these reduced the activity of FII, but changes at the 3′ end of the fragment (x3′) caused a complete loss of enhancer blocking activity. Likewise, deletion of 10 bp from both ends (ΔF) or a reversal of the sequence 5′→3′ (rev) resulted in substantial losses in activity.

Armed with this information, we searched for a protein that bound to FII with a competition profile that matched the sequence specificity observed in the enhancer blocking assay. To do this, we made nuclear extracts from the human erythroleukemic cell line K562 (the cell line in which the enhancer blocking assay is performed) and from adult chicken red blood cells (since this insulator is a chicken element). Identical patterns were obtained with these two extracts in a gel mobility-shift assay (Figure 3B). In each case two major complexes were observed when the extract was incubated with a 60 bp probe spanning footprint II. The upper complex could be supershifted with an antibody against Sp1 and was competed by a 100-fold excess of an unlabeled Sp1 consensus–binding site. We conclude that this complex contains Sp1. In contrast, the lower complex was neither supershifted by anti-Sp1, nor was its binding influenced by an excess of Sp1 consensus–binding site. More importantly, the affinity of the lower complex for various mutants of FII paralleled, with striking accuracy, the enhancer blocking activities of those same fragments (Figure 3C). The slight deviations we observed could arise from differential effects of the mutations on the binding of the insulator protein and Sp1 which, as we showed earlier, complicate interpretation of the in vivo results.

A Single Protein Is Responsible for the Sequence Specificity of Enhancer Blocking

Probing a blot of nuclear proteins with labeled FII revealed a single FII-specific DNA-binding protein with an apparent size of ~140 kDa (Figure 4A). The affinity of this protein for FII variants was identical to that observed in the gel-shift assay and was also in good agreement with the enhancer blocking data (Figure 3C and 4A). We purified this protein from chicken red blood cell nuclear

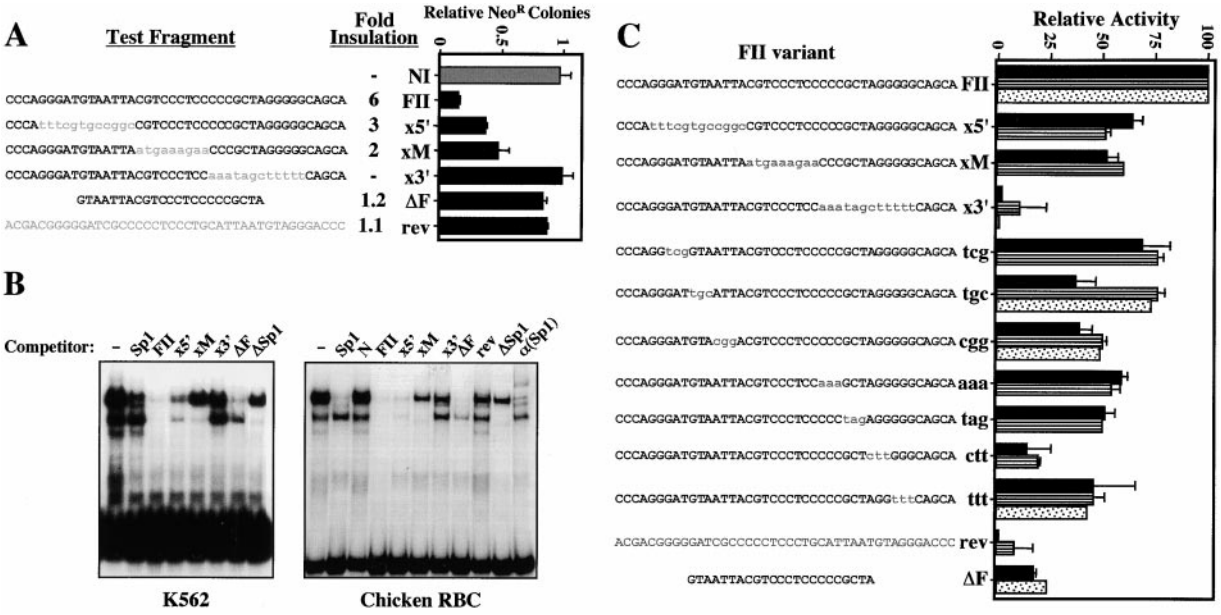


Figure 3. Sequence Specificity of Enhancer Blocking and Nuclear Factor Binding by FII

(A) The enhancer blocking capacity of the indicated FII variants was measured in the colony assay.

(B) Gel mobility shift assays of FII and nuclear extracts from human K562 and chicken red blood cells (RBC). Cold competitors as indicated were added at a 100-fold molar excess in these experiments (sequences are in [A]; N is a nonspecific oligo, α(Sp1) indicates addition of Sp1 antiserum).

(C) Comparison of the capacity of the indicated FII mutants to (i) act as insulators in the colony assay (black), (ii) compete with FII for binding to a candidate insulator protein in gel shift (striped), and (iii) compete with FII for binding to CTCF in a Southwestern assay (stippled). Data were normalized with FII activity considered as 100% in each assay.

extract by conventional chromatography. Throughout the purification, the elution profiles of the FII binding activities were identical in gel-shift and Southwestern assays (data not shown). This protein bound tightly to S, CM, and hydroxyapatite columns, and eluted with a peak at ~330 kDa on gel filtration (Figure 4B). Coomassie staining of gels from the final hydroxyapatite fractions revealed a single protein with an apparent molecular weight of ~140 kDa corresponding to the position of the FII Southwestern activity. The sequences of four internal peptides (Figure 4C) from the 140 kDa DNA-binding component of our final purified fraction all perfectly matched the predicted sequence of a previously cloned 11–zinc finger DNA-binding protein, CTCF (Klenova et al., 1993; Filippova et al., 1996).

Consistent with this identification, in vitro–translated CTCF binds to FII with a sequence specificity identical to that observed in our gel-mobility shift and enhancer blocking assays (Figure 5). As is expected, this protein also binds to other previously characterized CTCF sites (Figure 5, lanes 9–11), and these sites also act as enhancer blockers in our assay (Figure 5). Alignment of these CTCF sites with FII reveals a conserved region that has been shown to be critical for binding of CTCF to these other sites (Filippova et al., 1996; Burcin et al., 1997; Vostrov and Quitschke, 1997). We note that it was mutation of this conserved 3′ sequence that completely abrogated CTCF binding and enhancer blocking in our assays (see x3′ in Figure 3 and alignments in 6A).

Conservation of Sequence among Vertebrate Insulators

Because CTCF is highly conserved among vertebrates, we examined whether CTCF sites are present in other vertebrate insulators. Two such elements have recently been described. A 1.4 kb fragment found in the intergenic spacer region of the ribosomal RNA genes of *Xenopus laevis*, termed the repeat organizer (RO), has been shown to prevent enhancer action in a directional manner (Robinett et al., 1997). The 3′ half of this sequence is composed of seven tandem repeats of an ~100 bp GC-rich sequence (Labhart and Reeder, 1987). This sequence bears significant homology with CTCF sites including FII (Figure 6B). Moreover, a DNA fragment spanning one repeat unit of RO binds specifically, albeit weakly, to CTCF in vitro (Figure 7A). In our in vivo assay, the full-length RO element conferred considerable enhancer blocking activity, and a single copy of the 100 bp repeat from this element had weak enhancer blocking activity on its own (Figure 7B).

Another vertebrate insulator, BEAD-1, is a 1.6 kb enhancer-blocking element derived from the human *T cell receptor* α/δ locus (Zhong and Krangel, 1997). Best-fit alignment of this element with various CTCF sites revealed a good match between FII and a sequence roughly at the center of this element (BEAD-A in Figure 6C). In fact, a DNA fragment containing this region also bound specifically to purified chicken CTCF (Figure 7A). Consistent with these observations, both full-length

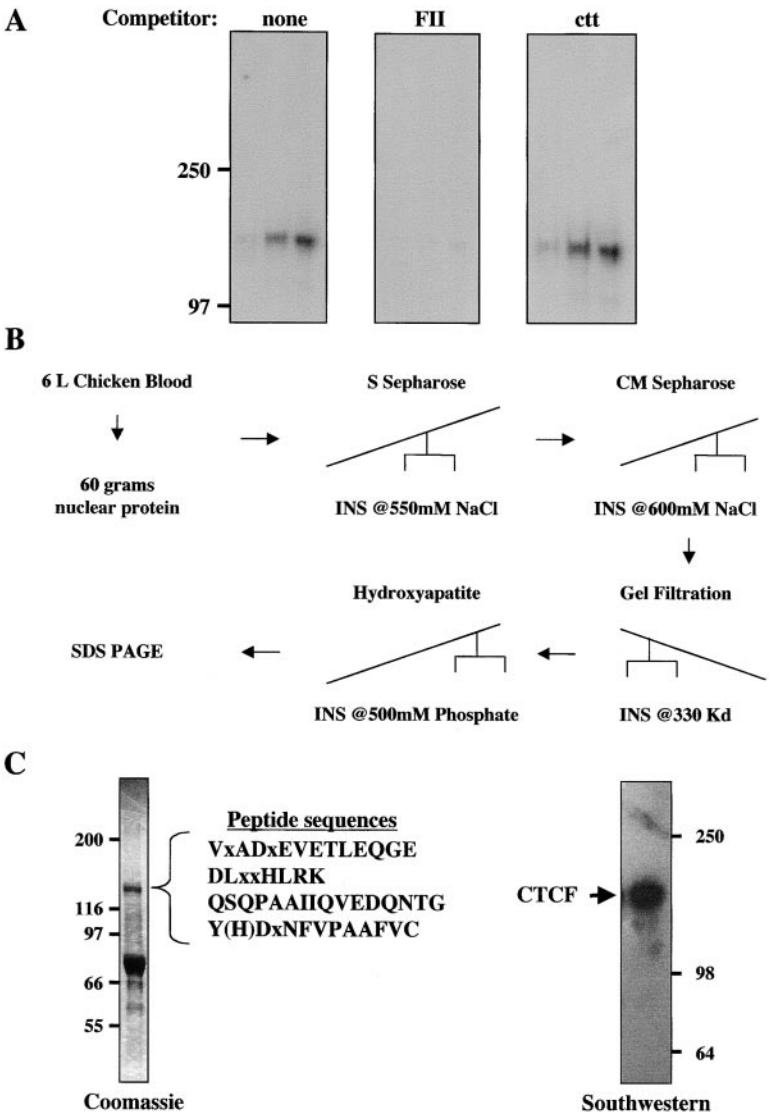


Figure 4. Purification of an FII-Binding Factor

(A) Southwestern assay reveals sequence-specific FII binding at ~140 kDa apparent molecular weight in protein fractions obtained during different stages of purification. Identical blots were probed simultaneously with labeled FII in the presence of a 50-fold excess of the indicated unlabeled cold competitor.

(B) An outline of the scheme used to purify the FII-binding factor.

(C) A representative example of a Coomassie-stained gel of the purified fractions eluted from the hydroxyapatite column is shown on the left with the internal peptide sequences obtained from the indicated band shown. The result of a Southwestern assay of FII binding to this fraction is shown in the right panel.

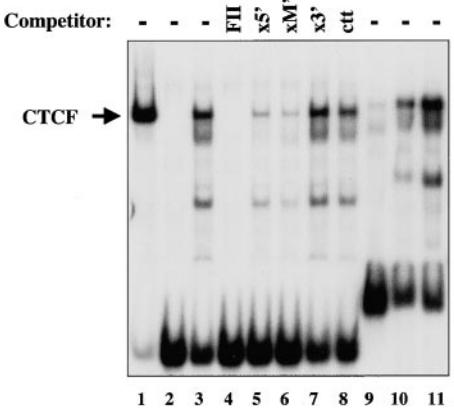
BEAD-1 and the CTCF-binding BEAD-A element defined here were effective enhancer-blocking elements in our assay (Figure 7B). Furthermore, deletion of the BEAD-A sequence from BEAD-1 largely eliminated the activity of the larger element.

Discussion

A major defining property of insulators is their ability to interfere with enhancer–promoter interaction when placed between them. Our laboratory previously has shown that a 1.2 kb element at the 5′ end of the chicken β-*globin* locus has such enhancer blocking activity. Here we identify a small DNA sequence motif within this insulator that is sufficient to account for most of its ability to block enhancers. A 42 bp fragment containing the motif was able to suppress enhancer activity in a directional manner about as well as the full 1.2 kb element from which it was derived. Although the fragment contains binding sites for Sp1 and the yeast α2 repressor, mutating these sites had no effect on blocking activity. However, mutations of the 3′ end of this site did abolish enhancer blocking.

When FII was used as a probe in gel retardation experiments with crude extracts, two major complexes were observed. One of these was attributable to Sp1. The other had properties implicating it in insulating activity: it was competed by any DNA that was also active in the enhancer blocking assay, but not by any of the inactive mutated sequences. Similar gel shift patterns were obtained with extracts from chicken erythrocyte nuclei and the human erythroleukemia line K562, in which the enhancer blocking assays were carried out. We used this result to purify the protein responsible for this complex and found that it was identical to a known protein, CTCF. Consistent with this, in vitro–translated CTCF bound FII with sequence specificity identical to the original complex. Furthermore, the original complex was supershifted with an antibody against CTCF.

CTCF is an 82 kDa protein with 11 zinc fingers (Filippova et al., 1996) and is characterized by an unusually extensive DNase I footprint (51 bp) when bound to its



CTCF site	Fold insulation
FII	6±2
myc	4±2
lys	4±2
Apβ	7±1

Figure 5. FII Binding and Enhancer Blocking by CTCF

Purified FII-binding factor (lane 1) and in vitro–translated CTCF (lanes 2–11) have identical specificity for FII (compare to Figure 3) and identical migration in a gel shift assay when bound to FII (lanes 2–8) or previously characterized CTCF sites from the chicken c-*myc* promoter (lane 9), the chicken *lysozyme* promoter (lane 10) or the human *amyloid β protein* promoter (lane 11). The right table summarizes the capacity of these CTCF sites to act as enhancer blockers in the colony assay. Data are the average of two independent measurements.

site on DNA, consistent with an involvement of several fingers in typical binding sites. It migrates anomalously on acrylamide/SDS gels, which accounts for the discrepancy in apparent molecular weight (Klenova et al., 1997). Studies of CTCF in other systems suggest that it can play a variety of regulatory roles. It binds to the promoter of the amyloid β protein precursor and causes transcriptional activation (Vostrov and Quitschke, 1997), but when it interacts with sites in the c-*myc* oncogene it causes repression (Filippova et al., 1996). It is also capable of acting in synergy with certain thyroid hormone receptor binding sites both in repression and in T3 induction (Baniahmad et al., 1990). Not all of the 11 zinc fingers of the protein are involved in binding to the sites that have been examined so far. Furthermore, different sites employ partially different subsets of fingers to contact the DNA (Filippova et al., 1996). One can imagine that the characteristics of the binding site would have a large influence on the conformation of the protein, the nature of its interactions with cofactors, and its ultimate biological effect.

The best characterized insulators are found in *Drosophila*. The directional enhancer blocking activity of these elements requires recognition of particular insulator sequences by specific DNA-binding proteins. In *Drosophila*, three seemingly unrelated DNA-binding proteins are required for the action of distinct insulators (Spana et al., 1988; Holdridge and Dorsett, 1991; Geyer and Corces, 1992; Zhao et al., 1995; Ohtsuki and Levine, 1998). These proteins act, presumably in concert with other factors, to somehow restrict the action of an enhancer in a directional manner (Kellum and Elgin, 1998; Bell and Felsenfeld, 1999; Udvardy, 1999). While the mechanism through which insulator action is achieved remains unknown, sequence-specific binding by these proteins is a necessary step in defining this process. Our data strongly support the notion that CTCF is capable of directing enhancer blocking in vertebrates. First, by whittling down the chicken β-globin insulator to a minimal active element, we identified a CTCF site that plays an essential role in that element's activity. Accurate appraisal of the full enhancer blocking capacity of this element was complicated by the presence of Sp1 sites, whose activity apparently masked that of CTCF in our assay. By mutating the Sp1 sites in this region (Figure 2C, FII/III-ΔSp1*), however, the "full" 9-fold enhancer

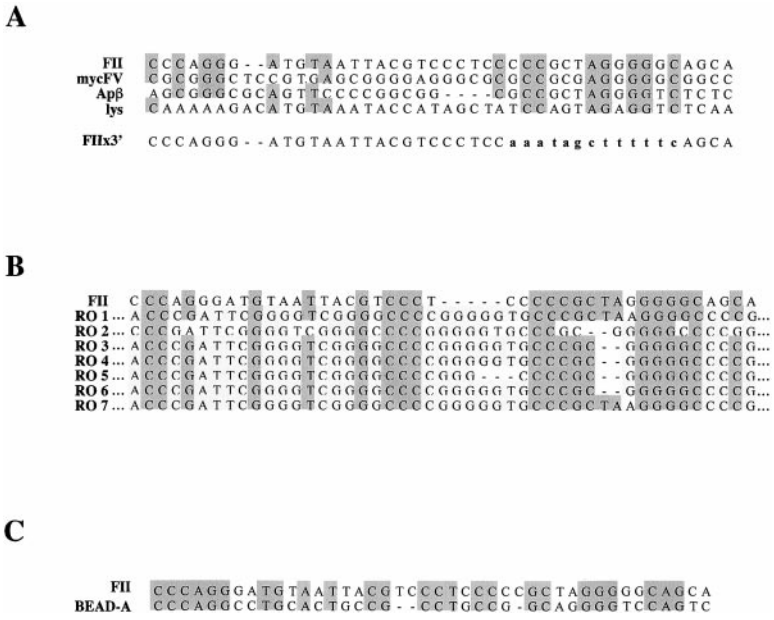


Figure 6. Sequence Homologies among CTCF Sites and Vertebrate Insulators

(A) Alignment of FII with other known CTCF sites reveals a conserved 3′ region which corresponds to the sequence altered in the x3′ mutant (see Figure 3).

(B) Alignment of the 100 bp repeats of the *Xenopus* RO element and FII.

(C) Alignment of FII with a homologous site (BEAD-A) in the BEAD-1 element from the human *T cell receptor* α/δ locus.

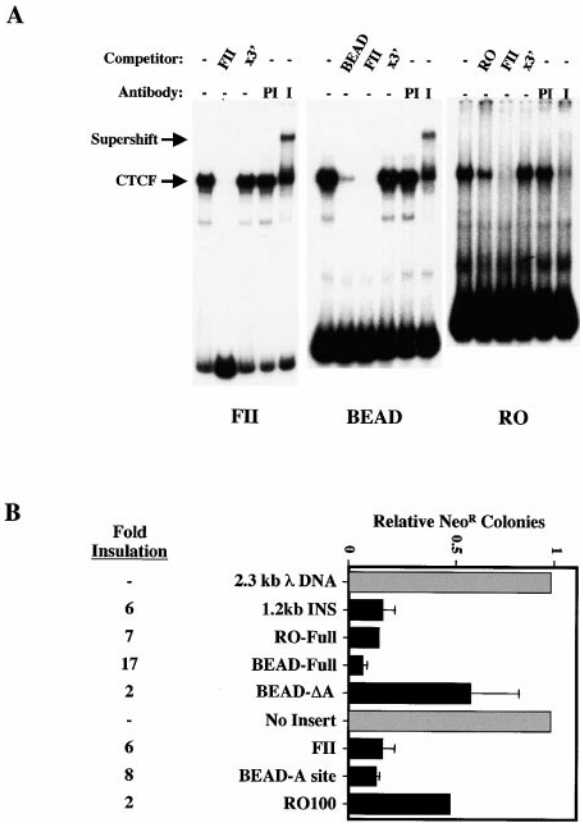


Figure 7. Conservation of Sequence-Specific Enhancer Blocking Activity among Vertebrate Insulators

(A) Gel mobility shift assays with FII, RO542–90, and BEAD-A70 as probes reveal sequence-specific binding to partially purified CTCF. An antibody raised against a C-terminal peptide of CTCF specifically supershifts all three complexes. PI indicates the addition of preimmune IgY and I indicates the addition of anti-CTCF (C-terminal) IgY.

(B) Enhancer blocking activities of vertebrate insulators. These data are the average of at least two independent experiments with the exception that the data for the RO elements is from a single determination.

blocking capacity of this element was revealed. This activity is equal to that observed with the entire 1.2 kb insulator (Chung et al., 1993). Our conclusions are further supported by the observation that, in vivo, the enhancer blocking activity of various mutated FII fragments accurately parallels the capacity of these fragments to bind CTCF in vitro. Finally, in every case we examined, binding sites for CTCF were capable of directional enhancer blocking in vivo. Taken together, our results show that CTCF binding sites are necessary and sufficient for enhancer blocking activity in our assay. The fact that several DNA-binding proteins have been implicated in insulator function in *Drosophila* may indicate that enhancer-blocking proteins other than CTCF will ultimately be found in vertebrates. However, the presence of CTCF sites in each of the vertebrate loci shown thus far to have this activity is strong evidence for a conserved role of CTCF in insulator function in vivo.

As we have pointed out in the Introduction, the β-globin 5′ insulator element shares with the *Drosophila* insulators the additional ability to protect against position effects. When two copies of the entire 1.2 kb fragment containing the β-globin 5′ HS4 are placed on either side of a stably integrated reporter gene, the reporter is protected both against variation in expression from one line to another, and also against extinction of expression over a period of at least 40–80 days in culture (Pikaart et al., 1998). Preliminary results (F. Recillas-Targa et al., unpublished) indicate that this activity depends upon sequences other than FII within the insulator. The complete activity of the β-globin 5′ insulator element is thus likely to involve multiple components.

We find that the enhancer blocking activity of the 5′ β-globin insulator is dependent upon CTCF, and that similar sequences are present in the two other vertebrate insulators. The first of these is the BEAD-1 element found in the human *T cell receptor* (*TCR*) α/δ locus (Zhong and Krangel, 1997). BEAD-1, which has strong directional enhancer blocking properties, is located between *TCR*δ gene segments and *TCR*α joining gene segments. It has been proposed that BEAD-1 prevents a δ-specific enhancer from acting on the α genes early in T cell development. We have shown here that BEAD-1 contains a CTCF-binding site and that this site is responsible for a large portion of the observed enhancer blocking activity. We have also examined the enhancer blocking activity of an element that derives from the 5′-boundary of the chicken *lysozyme* gene (Stief et al., 1989). Consistent with the findings of Stief et al., we found that this element conferred considerable enhancer blocking activity in our assay and that this element contains (at least) two bona fide binding sites for CTCF, both of which are independently capable of enhancer blocking in our assays (data not shown).

A specialized insulator element has been described within the *Xenopus* ribosomal RNA repeats (Robinett et al., 1997). A 100 bp repeating sequence within this element has significant homology with FII, binds CTCF, and confers enhancer blocking in our assay. In the system described by Robinett et al., enhancer blocking by the full RO element was only revealed in constructions where the relative position of enhancer, promoter, and insulator elements mimicked the in vivo arrangement of these elements. In our assay, this element was effective in enhancer blocking, whereas analogous constructions failed to reveal the enhancer blocking capacity of this element in *Xenopus* oocytes. These discrepancies may be explained by differences in the enhancer and promoter functions in these systems or they may reflect species or cell type–specific differences in the contribution of CTCF, or other factors, to the enhancer blocking capacity of RO. To date, a *Xenopus* homolog of CTCF has not been identified. However, given the fact that this protein is highly conserved in other vertebrates (Filippova et al., 1996), it is reasonable to suppose that such a homolog exists.

There seems little doubt that CTCF plays a major role in the enhancer blocking activity of all of these elements. Our focus on FII in the β-globin insulator led us to this conserved attribute of vertebrate insulators. A minor contribution to enhancer blocking is also made by FIII. This may arise from a weak CTCF consensus in this site. Taken together, our results suggest a conserved and perhaps widely used function of insulators, involving CTCF, in the maintenance of distinct regulatory regions. Indeed, recent results in our laboratory (N. Saitoh et al., unpublished) show that the 3′ end of the chicken β-*globin* locus is marked by a hypersensitive site with

similar properties to 5′HS4 and that it also contains a CTCF-binding site. This element is located between the globin genes and a nearby gene encoding an odorant receptor (Bulger et al., 1999) further substantiating the nature and likely function of these boundary elements. It may be that in some situations, where enhancer blocking activity is all that is required, a CTCF-binding site alone is sufficient, whereas in the case of a permanent chromatin domain boundary, such as that found at the 5′ end of the chicken β-*globin* locus, additional components are involved. We surmise that, even in those cases where only CTCF sites are present, the activity of CTCF requires the participation of other proteins, just as the directional enhancer blocking activity of the suppressor of Hairy-wing protein involves interaction with the Mod(mdg4) protein (Gerasimova et al., 1995; Gdula et al., 1996; Georgiev and Kozycina, 1996; Gdula and Corces, 1997; Gerasimova and Corces, 1998). We are currently exploring the possibility that proteins that copurified with CTCF in our preparations may participate in insulator function.

We have demonstrated that in our system a CTCF site must be located between enhancer and promoter to influence expression. This result depends only on the position of the insulator; it can be inserted in either orientation with equal effect (data not shown). These properties must be reconciled with our partial understanding of how enhancers function. Various models have been proposed to account for enhancer blocking. They fall into two broad categories: steric models and tracking models (Kellum and Elgin, 1998; Bell and Felsenfeld, 1999; Udvardy, 1999). Steric mechanisms postulate that insulators partition enhancer and promoter into two separate domains that are inaccessible to each other. Tracking models suppose that some activating signal must travel along the DNA from enhancer to promoter, and that the insulator blocks this transmission. Our inability to distinguish among these models results, in part, from an incomplete understanding of how enhancers work.

We conclude that CTCF is likely to play a role in the enhancer blocking function of many insulator elements. In the case of the 5′-globin insulator, an independently regulated gene encoding a folate receptor has recently been identified 5′ of the globin locus (Prioleau et al., 1999). These genes are close enough to each other that the regulatory elements of the two loci might influence each other inappropriately in the absence of an insulator. A rather similar situation exists in the case of the *T cell receptor* locus (Zhong and Krangel, 1997) where the BEAD insulator may prevent inappropriate activity of an enhancer. The presence of CTCF sites in these quite different genetic loci implies that the role of such sites in the establishment of enhancer boundaries is likely to be a conserved and important component of gene regulation.

Experimental Procedures

Plasmid Constructions and Oligonucleotides

The plasmid pNI was the base plasmid for enhancer blocking assays. pNI was generated by replacing the SacI copy of the 1.2 kb insulator in pJC5-4 (Chung et al., 1993) with an AscI linker after digestion of this plasmid with Ecl136II. Fragments subcloned into the AscI site of this plasmid are located between the enhancer (mouse HS2) and the reporter ("γ-neo"). The following primers were used in PCR amplifications to generate fragments for cloning into the AscI site of pNI: AC1F, AGGCGCGCCTGGGAGCTCACGGGGAC AGCCCCC; AC1R, AGGCGCGCCTGGGAGCGCCGGACCGGAGCG GAG; AC2F, AGGCGCGCCGGCTCCGCTCCGGTCCGGCGCTCC; AC2R, AGGCGCGCCTGTCATTCTAAATCTCTCTTTCAGC; ΔIACF, AGGCGCGCCGCCCCCAGGGATGTAATTACGTCC; ΔIIacF, AGCCC CCCCCCAAA GCCCCCAGGGATGGGGGCAGCAGCGAGCCGC; ΔIIacR, GGCGGCTCGCTGC TGCCCCCATCCCTGGGGGCTTTGGGG GGGGGC; ΔIIIACF, CCGAGCCGGCAGCGTGCGGGGACAG; ΔIIIACR, CCCGCACGCTGCCGGCTCGGCGGACCGGAGCGGAGCCCCG; ΔIVACF, CCTCTGAACGCTTCTCGCTGCTCTT; ΔIVACR, CAGCGAG AAGCGTTCAGAGGCCTTCCCCGTGCCCGGGCTG; ΔVACR, AGGC GCGCCGCCCAGGTGTCTGCAGGCTCAAAGAGC; BEADascF, AGG CGCGCCGAATTCCAGAAATCTTTGATTTCAGATGCT; BEADascR, AGGCGCGCCGGATCCCACTCTTAGCCATTATACTGCATTG; BEADΔAF, TGAGCATCTTCAGGGCCCCTGGATTCCATTTCAGAGCTTCC GGTTCTC; BEADΔAR, ATCCAGGGGCCCTGAAGATGCTCA. The core, FI/FII, FIII/IV/V, ΔFI, and ΔFV were generated by PCR using the plasmid p501 (Reitman and Felsenfeld, 1990) as a template and the primer pairs AC1F/AC2R, AC1F/AC1R, AC2F/AC2R, ΔIACF/AC2R, and AC1F/ΔVACR, respectively. Deletions of FII, FIII, and FIV from the core were accomplished by two-step, overlapping PCR. For each deletion, a pair of intermediate fragments was generated by PCR in separate reactions using p501 as the template and the primer pairs AC1F/ΔIIacR, ΔIIacF/AC2R, AC1F/ΔIIIACR, ΔIIIACF/AC2R, AC1F/ΔIVACR, and ΔIVACF/AC2R. The products of each of these reactions were gel purified, mixed pairwise to generate the appropriate templates, and the final products were amplified with AC1F/AC2R. The full-length BEAD-1 fragment was generated by PCR from K562 genomic DNA with primers BEADascF and BEADascR. The fragment BEADΔA was generated in a two-step, overlapping PCR reaction, first using the BEAD-1 fragment as a template and the primers BEADascF/BEADΔAR and BEADΔAF/ BEADascR in separate reactions, then mixing the gel-purified products of these reactions with primers BEADascF and BEADascR to generate the final product by PCR. A 1.6 kb fragment containing the full-length RO element was subcloned into Ecl136II cut pJC5-4 after liberation of this fragment from p0,1 (Robinett et al., 1997) by digestion with Ecl136II and PvuII. All other enhancer-blocking fragments were generated by direct synthesis of the appropriate complementary oligonucleotides on an ABI 394 DNA synthesizer. The top strands of these were: FII/III, AGGCGCGCCGGGATGTAATTACGTCCCTCCCC CGCTAGGGGGCAGCAGCGAGCCGCCCGGGGCTCCGCTCCGGT CCGGCGCTCCCCCCGCATCCCCGAGGGCGCGCCT; FII/III-ΔSp1*, AGGCGCGCCGGGATGTAATTACGTCCCTAACCCGCTAGGGGGC AGCAGCGAGCCGAACGGGGCTCCGCTCCGGTCCGGCGCTAACC CCGCATCCCCGAGGGCGCGCCT; FII/III-Δα2, AGGCGCGCCGGGA TGTACGTCCCTCCCCCGCTAGGGGGCAGCAGCGAGCCGCCCGG GGCTCCGCTCCGGTCCGGCGCTCCCCCCGCATCCCCGAGGGC GCGCCT; Δ spacer, AGGCGCGCCCCCAGGGATGTAATTACGTCC CTCCCCCGCTAGGGGGCAGCACCGGTCCGGCGCTCCCCCCGC ATCCCCGAGCCGGGGCGCGCCT; ΔIIN, AGGCGCGCCGGGGGCA GCAGCGAGCCGCCCGGGGCTCCGCTCCGGTCCGGCGCTCCCC CCGCATCCCCGAGGGCGCGCCT; ΔIIIN, AGGCGCGCCCCAAAGC CCCCAGGGATGTAATTACGTCCCTCCCCCGCTAGGGGGCAGCA GCGAGCCGCCCGGGGCTCCGCGGCGCGCCT; FII, AGGCGCGCC CCCAGGGATGTAATTACGTCCCTCCCCCGCTAGGGGGCAGCAG GCGCGCCT; FIII, AGGCGCGCCCCGGTCCGGCGCTCCCCCCGC ATCCCCGAGCCGGGGCGCGCCT; gypsy-3, AGGCGCGCCAAAAT ACATTGCATACCCTCTTTTAATAAAAAATATTGCATACGTTGACGA AACAAATTTTCGTTGCATACCCAATAAAAGGCGCGCCT; mycFV, AGGCGCGCCGGGGGGGGGCACGGAGCCCCTCGGCCGCCCCCT CGCGGCGCGCCCTCCCCGCTCACGGAGCCCGCGCGGAGCCGG GGGCGAGGCGCGCC; lys, AGGCGCGCCTTTAGCTGCATTTGACA TGAAGAAATTGAGACCTCTACTGGATAGCTATGGTATTTACATGT CTTTTTGCTTAGTTACTAGGCGCGCC; Apβ, AGGCGCGCCCCCTC CCGGCGCGAGCGGGCGCAGTTCCCCGGCGGCGCCGCTAGGGG TCTCTCTCGGGTGCCGAGCGGGGTGGGCCGGATAGGCGCGCC; RO100, AGGCGCGCCGGGGACCCGATTCGGGGTCGGGGCCCCG GGGGTGCCCGCTAAGGGGCCCCGGGGGGCCCTCCCGGCGAAG AGGGGCCCATTGGCGCGCCT; BEAD-A, AGGCGCGCCGTGGAAG AGGGATGTTGAGGGCCCAGGGGCTGCCTTGCCGGTGCATTGGC

TGCCCAGGCCTGCACTGCCGCCTGCCGGCAGGGGTCCAGTCC ACGAGACCCAGCTCCCTGCTGGCGGAAGGGCGCGCCT. All FII mutants were identical to FII except for those bases indicated in lowercase in Figure 3C. For use in the enhancer blocking assay, complementary single-stranded oligonucleotides were purified by denaturing PAGE, annealed, and subcloned into pNI. The FII site was also generated with NdeI sites at its ends for cloning upstream of the enhancer in pNI to generate FII-UP. To generate FII-DOWN, FII was digested out of pNI-FII, the ends were flushed with Klenow, and XbaI linkers (New England Biolabs) were added for cloning into the XbaI site of pNI.

Enhancer Blocking Assay

Enhancer blocking assays were performed as previously described (Chung et al., 1993, 1997). Briefly, 20 μg of each construct was linearized by SalI digestion, phenol-chloroform extracted, ethanol precipitated, and quantified by UV absorption. Twenty nanograms of each DNA was then electroporated into 1×10^7 K562 cells and, after 24 hr of recovery, cells were plated in soft agar with geneticin at 750 μg/ml. Colonies were counted after 3 weeks of selection and the colony number was normalized to that obtained with pNI or pJC3-4 (Chung et al., 1993).

DNase I–Hypersensitive Site Analysis

Nuclei were isolated from adult chicken red blood cells essentially as described previously (Bresnick and Felsenfeld, 1994) except that 0.2 mM EGTA was included in all buffers. After incubation of the nuclei with varying concentrations of DNase I for 5 min at room temperature, the reaction was terminated by the addition of SDS and genomic DNA was purified. To map precisely the position of HS4, DNase I digested and undigested genomic DNAs (10 μg) were further digested with StyI to generate an ~1 kb parent fragment that spanned the insulator core. This DNA was then digested with the enzymes indicated in Figure 1A. Digested DNA was resolved on a 1.3% agarose gel and subjected to Southern blotting by standard techniques using a 503 bp StyI-SacI fragment of p501 (Reitman and Felsenfeld, 1990) as a probe.

DNA Binding Assays

All DNA binding assays were carried out in a binding buffer composed of 20 mM HEPES (pH 7.9), 150 mM KCl, 5 mM $MgCl_2$, 1 mM DTT. For gel mobility shift assays, DNA binding was carried out at room temperature for 30 min in binding buffer plus 5% glycerol, 20–40 fmol of end-labeled probe, poly-dI/dC at 50–100 μg/ml, 0.5% Triton X-100, and 1–5 μl of protein in 20 μl. Probes were oligonucleotide duplexes identical to those used for sub-cloning into the enhancer-blocking vector. The RO and BEAD sites, trimmed down for gel shifts, were RO542–90, 5′-GGCAGGGGACCCGATTCGGGGTCG GGGCCCCCGGGGGTGCCCGCTAAGGGGCCCCGGGGGGCCCTC CCGGCGAAGAGGGGCCCATTGGGA and BEADA-70, 5′-TGCATTG GCTGCCCAGGCCTGCACTGCCGCCTGCCGGCAGGGGTCCAGTC CACGAGACCCAGCTCCCTGC. Cold competitor duplexes were added simultaneously with labeled probes at 50-fold (Figures 3C, 5, and 7A) or 100-fold (Figure 3B) molar excess. Supershifts were carried out by preincubating the appropriate proteins in binding buffer for 2 hr at 0°C with an antibody raised against a C-terminal peptide (APNGDLTPEMILSMMD) of CTCF or against Sp1 (Santa Cruz Biotechnology), followed by a 30 min 23°C incubation with DNA. In the RO gel shifts in Figure 7A, 100 μg/ml poly-dG/dC (Roche Molecular Biochemicals) was substituted for poly-dI/dC. For Southwestern assays, proteins were TCA precipitated, resolved by SDS-PAGE, transferred to PVDF, and then denatured and renatured by successive 10 min incubations in binding buffer supplemented with guanidine hydrochloride at 4.8, 3, 1.5, and 0.75 molar. After a further 10 min wash in binding buffer, the blots were blocked in binding buffer plus 5% nonfat dry milk for 16 hr at 4°C. An FII probe was generated for Southwestern assays by annealing a full-length top strand (CCCAGGGATGTAATTACGTCCCTCCCCCGCTAGGGGGCA GCAGGCGCGCCT) to a short 3′ complementary primer (AGGCGCG CCTGCTGC). This partial duplex was extended with Klenow in the presence of [α-^{32}P]dCTP resulting in a probe identical to that used in gel-shift assays, but with 10 labeled phosphates per molecule. Blots were probed for 3 hr at room temperature in binding buffer supplemented with 0.25% nonfat dry milk, 5 μg/ml poly-dI/dC, and 3 pmol of labeled probe in a final volume of 20 ml, washed three times for 10 min in the same buffer without probe and exposed to film. In the example shown in Figure 4A, samples from an early pilot purification were loaded as follows: fractions of a large S sepharose, second small S sepharose, and subsequent Q sepharose column were loaded from left to right in each of the panels shown and probed as indicated in the figure legend.

Protein Purification and Translation

Nuclear extracts from K562 cells and whole chicken blood were prepared essentially as previously described (Evans et al., 1988). For purification of the FII-binding protein, nuclei were prepared from 6 liters of whole chicken blood (Pelfreez Biologicals) and extracted in buffer C: 20 mM HEPES (pH 7.9), 420 mM NaCl, 5 mM $MgCl_2$, 0.2 mM EDTA, and 1 mM DTT. The resulting extract was diluted to 150 mM NaCl/20% glycerol in the same buffer and fractionated on a 500 ml SP sepharose column (Pharmacia) using a 0.15–1 M NaCl linear gradient. Active fractions were pooled, diluted to ~150 mM NaCl, and fractionated on a 25 ml CM sepharose column with a 0.15–1 M NaCl gradient. These fractions were pooled and loaded onto a 2.6/60 cm Sephacryl S-300 gel filtration column (Pharmacia) preequilibrated with 20 mM HEPES (pH 7.9), 150 mM NaCl, 5 mM $MgCl_2$, 0.2 mM EDTA, and 1 mM DTT. Active fractions were pooled, dialyzed into 10 mM potassium phosphate (pH 8.0), 150 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 20% glycerol, and loaded onto a 25 ml Macro-Prep ceramic hydroxyapatite column (Bio-Rad). This column was eluted with a 10–800 mM phosphate gradient at pH 8.0. Throughout the isolation all buffers were supplemented with 1 mM PMSF, 0.7 μg/ml pepstatin, and 0.5 μg/ml leupeptin and maintained at 4°C. Fractions pooled from the gel filtration, and all subsequent buffers, were supplemented with 40 μg/ml bestatin and 200 μg/ml AEBSF. For peptide sequencing, 1 ml of a final active fraction (representing ~1/10th of our final yield and ~5 μg of purified ~140 kDa protein) was TCA precipitated, resolved on a 7% Tris-acetate SDS-PAGE (Novex), transferred to PVDF, stained with amido black, and internal protein sequence was obtained at the Rockefeller University Protein/DNA Technology Center by in situ digestion with endoproteinase Lys-C followed by HPLC purification of individual peptides and Edman sequence determination of each peptide. In vitro–translated human CTCF was obtained using the plasmid p4B7.1 (obtained from V. Lobanenkov) as a template for in vitro transcription by T7 polymerase according to the manufacturer's instructions (Ambion, "Message Machine"), followed by in vitro translation of the resulting RNA in a nuclease-treated rabbit reticulocyte system (Promega).

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References

Anderson, G.M., and Freytag, S.O. (1991). Synergistic activation of a human promoter in vivo by transcription factor Sp1. Mol. Cell. Biol. *11*, 1935–1943.

Baniahmad, A., Steiner, C., Kohne, A.C., and Renkawitz, R. (1990). Modular structure of a chicken lysozyme silencer: involvement of an unusual thyroid hormone receptor binding site. Cell *61*, 505–514.

Bell, A.C., and Felsenfeld, G. (1999). Stopped at the border: boundaries and insulators. Curr. Opin. Genet. Dev. *9*, 191–198.

Bresnick, E.H., and Felsenfeld, G. (1994). Dual promoter activation by the human beta-globin locus control region. Proc. Natl. Acad. Sci. USA *91*, 1314–1317.

Bulger, M., von Doorninck, J.H., Saitoh, N., Telling, A., Farrell, C., Bender, M.A., Felsenfeld, G., Axel, R., and Groudine, M. (1999). Conservation of sequence and structure flanking the mouse and

human beta-globin loci: the beta-globin genes are embedded within an array of odorant receptor genes. Proc. Natl. Acad. Sci. USA *96*, 5129–5134.

Burcin, M., Arnold, R., Lutz, M., Kaiser, B., Runge, D., Lottspeich, F., Filippova, G.N., Lobanenkov, V.V., and Renkawitz, R. (1997). Negative protein 1, which is required for function of the chicken lysozyme gene silencer in conjunction with hormone receptors, is identical to the multivalent zinc finger repressor CTCF. Mol. Cell Biol. *17*, 1281–1288.

Chung, J.H., Whiteley, M., and Felsenfeld, G. (1993). A 5′ element of the chicken β-globin domain serves as an insulator in human erythroid cells and protects against position effect in Drosophila. Cell *74*, 505–514.

Chung, J.H., Bell, A.C., and Felsenfeld, G. (1997). Characterization of the chicken beta-globin insulator. Proc. Natl. Acad. Sci. USA *94*, 575–580.

Dorsett, D. (1993). Distance-independent inactivation of an enhancer by the suppressor of Hairy-wing DNA-binding protein of Drosophila. Genetics *134*, 1135–1144.

Evans, T., Reitman, M., and Felsenfeld, G. (1988). An erythrocyte-specific DNA-binding factor recognizes a regulatory sequence common to all chicken globin genes. Proc. Natl. Acad. Sci. USA *85*, 5976–5980.

Filippova, G.N., Fagerlie, S., Klenova, E.M., Myers, C., Dehner, Y., Goodwin, G., Neiman, P.E., Collins, S.J., and Lobanenkov, V.V. (1996). An exceptionally conserved transcriptional repressor, CTCF, employs different combinations of zinc fingers to bind diverged promoter sequences of avian and mammalian c-myc oncogenes. Mol. Cell Biol. *16*, 2802–2813.

Gdula, D.A., and Corces, V.G. (1997). Characterization of functional domains of the su(Hw) protein that mediate the silencing effect of mod(mdg4) mutations. Genetics *145*, 153–161.

Gdula, D.A., Gerasimova, T.I., and Corces, V.G. (1996). Genetic and molecular analysis of the gypsy chromatin insulator of Drosophila. Proc. Natl. Acad. Sci. USA *93*, 9378–9383.

Georgiev, P., and Kozycina, M. (1996). Interaction between mutations in the suppressor of Hairy wing and modifier of mdg4 genes of Drosophila melanogaster affecting the phenotype of gypsy-induced mutations. Genetics *142*, 425–436.

Gerasimova, T.I., and Corces, V.G. (1998). Polycomb and trithorax group proteins mediate the function of a chromatin insulator. Cell *92*, 511–521.

Gerasimova, T.I., Gdula, D.A., Gerasimov, D.V., Simonova, O., and Corces, V.G. (1995). A Drosophila protein that imparts directionality on a chromatin insulator is an enhancer of position-effect variegation. Cell *82*, 587–597.

Geyer, P.K., and Corces, V.G. (1992). DNA position-specific repression of transcription by a Drosophila zinc finger protein. Genes Dev. *6*, 1865–1873.

Hagstrom, K., Muller, M., and Schedl, P. (1996). Fab−7 functions as a chromatin domain boundary to ensure proper segment specification by the Drosophila bithorax complex. Genes Dev. *10*, 3202–3215.

Hebbes, T.R., Clayton, A.L., Thorne, A.W., and Crane-Robinson, C. (1994). Core histone hyperacetylation co-maps with generalized DNase I sensitivity in the chicken beta-globin chromosomal domain. EMBO J. *13*, 1823–1830.

Holdridge, C., and Dorsett, D. (1991). Repression of hsp70 heat shock gene transcription by the suppressor of hairy-wing protein of Drosophila melanogaster. Mol. Cell Biol. *11*, 1894–1900.

Kellum, R., and Elgin, S.C. (1998). Chromatin boundaries: punctuating the genome. Curr. Biol. *8*, R521–R524.

Kellum, R., and Schedl, P. (1991). A position–effect assay for boundaries of higher order chromosomal domains. Cell *64*, 941–950.

Kellum, R., and Schedl, P. (1992). A group of scs elements function as domain boundaries in an enhancer-blocking assay. Mol. Cell. Biol. *12*, 2424–2431.

Klenova, E.M., Nicolas, R.H., Paterson, H.F., Carne, A.F., Heath, C.M., Goodwin, G.H., Neiman, P.E., and Lobanenkov, V.V. (1993). CTCF, a conserved nuclear factor required for optimal transcriptional activity of the chicken c-myc gene, is an 11-Zn-finger protein differentially expressed in multiple forms. Mol. Cell. Biol. *13*, 7612–7624.

Klenova, E.M., Nicolas, R.H., U, S., Carne, A.F., Lee, R.E., Lobanenkov, V.V., and Goodwin, G.H. (1997). Molecular weight abnormalities of the CTCF transcription factor: CTCF migrates aberrantly in SDS-PAGE and the size of the expressed protein is affected by the UTRs and sequences within the coding region of the CTCF gene. Nucleic Acids Res. *25*, 466–474.

Labhart, P., and Reeder, R.H. (1987). DNA sequences for typical ribosomal gene spacers from Xenopus laevis and Xenopus borealis. Nucleic Acids Res. *15*, 3623–3624.

Lobanenkov, V.V., Nicolas, R.H., Adler, V.V., Paterson, H., Klenova, E.M., Polotskaja, A.V., and Goodwin, G.H. (1990). A novel sequence-specific DNA binding protein which interacts with three regularly spaced direct repeats of the CCCTC-motif in the 5′-flanking sequence of the chicken c-myc gene. Oncogene *5*, 1743–1753.

Ohtsuki, S., and Levine, M. (1998). GAGA mediates the enhancer blocking activity of the eve promoter in the Drosophila embryo. Genes Dev. *12*, 3325–3330.

Pikaart, M.J., Recillas-Targa, F., and Felsenfeld, G. (1998). Loss of transcriptional activity of a transgene is accompanied by DNA methylation and histone deacetylation and is prevented by insulators. Genes Dev. *12*, 2852–2862.

Prioleau, M.-N., Nony, P., Simpson, M., and Felsenfeld, G. (1999). An insulator element and condensed region separate the chicken β-globin locus from an independently regulated erythroid-specific folate receptor gene. EMBO J., *18*, 4035–4048.

Reitman, M., and Felsenfeld, G. (1990). Developmental regulation of topoisomerase II sites and DNase I–hypersensitive sites in the chicken beta-globin locus. Mol. Cell. Biol. *10*, 2774–2786.

Robinett, C.C., O'Connor, A., and Dunaway, M. (1997). The repeat organizer, a specialized insulator element within the intergenic spacer of the Xenopus rRNA genes. Mol. Cell. Biol. *17*, 2866–2875.

Sauer, R.T., Smith, D.L., and Johnson, A.D. (1988). Flexibility of the yeast alpha 2 repressor enables it to occupy the ends of its operator, leaving the center free. Genes Dev. *2*, 807–816.

Spana, C., Harrison, D.A., and Corces, V.G. (1988). The Drosophila melanogaster suppressor of Hairy-wing protein binds to specific sequences of the gypsy retrotransposon. Genes Dev. *2*, 1414–1423.

Stief, A., Winter, D.M., Stratling, W.H., and Sippel, A.E. (1989). A nuclear DNA attachment element mediates elevated and position-independent gene activity. Nature *341*, 343–345.

Udvardy, A. (1999). Dividing the empire: boundary chromatin elements delimit the territory of enhancers. EMBO J. *18*, 1–8.

Udvardy, A., Maine, E., and Schedl, P. (1985). The 87A7 chromomere. Identification of novel chromatin structures flanking the heat shock locus that may define the boundaries of higher order domains. J. Mol. Biol. *185*, 341–358.

Vostrov, A.A., and Quitschke, W.W. (1997). The zinc finger protein CTCF binds to the APBbeta domain of the amyloid beta-protein precursor promoter. Evidence for a role in transcriptional activation. J. Biol. Chem. *272*, 33353–33359.

Zhao, K., Hart, C.M., and Laemmli, U.K. (1995). Visualization of chromosomal domains with boundary element-associated factor BEAF-32. Cell *81*, 879–889.

Zhong, X.P., and Krangel, M.S. (1997). An enhancer-blocking element between alpha and delta gene segments within the human T cell receptor alpha/delta locus. Proc. Natl. Acad. Sci. USA *94*, 5219–5224.

Zhou, J., Barolo, S., Szymanski, P., Levine, M. (1996). The Fab–7 element of the bithorax complex attenuates enhancer−promoter interactions in the Drosophila embryo. Genes Dev. *10*, 3195–3201.