

A 5' Element of the Chicken β -Globin Domain Serves as an Insulator in Human Erythroid Cells and Protects against Position Effect in Drosophila

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Summary

We have characterized an element near the 5' boundary of the chicken β -globin domain that insulates a reporter gene from the activating effects of a nearby β -globin locus control region (5'HS2) when assayed in the human erythroid cell line K562. We show that the insulation mechanism is directional, that it operates at the level of transcription, and that it involves the alteration of chromatin structure over the promoter of the gene. The insulator has no significant stimulatory or inhibitory effects of its own. In transgenic Drosophila, the insulator protects the *white* minigene from position effects. The action of the insulator thus is not restricted to erythroid or mammalian cells, suggesting that such elements may serve an important and widely distributed function in the organization of chromatin structure.

Introduction

Over the past decade, we have learned a great deal about how tissue-specific genes are regulated at the level of transcription (reviewed by Muller et al., 1988; Ptashne, 1988; Thompson and McKnight, 1992). Such gene regulation is mediated through interaction between DNA regulatory sequences and a complex of transcriptional factors that are specific for that tissue type and gene. The higher order chromatin structure of these genes is also regulated in a tissue-specific manner (reviewed by van Holde, 1989). Transcriptionally active genes are typically embedded in a DNAase I-sensitive "domain" extending many kilobases to either side, consistent with the notion that the chromatin of the active domain is decondensed and easily accessible to trans-acting factors (Lawson et al., 1982; Groudine et al., 1983; Jantzen et al., 1986; Levy-Wilson and Fortier, 1989). This domain, in those tissues where the gene is not active, is condensed and therefore not accessible to trans-acting factors. It seems likely that this decondensation is an early event in determining whether the transcriptional factors will be able to interact with their binding sites in the domain (reviewed by van Holde, 1989; Jimenez et al., 1992a).

How are these domains organized? There is evidence that the genome is arranged in topologically isolated loops (Benyajati and Worcel, 1976). It has been proposed that these loops radiate from attachment points on the nuclear

matrix (Paulson and Laemmli, 1977; reviewed by Gasser and Laemmli, 1987; Garrard, 1990) and that these attachment points help to insulate the genes in one loop from the influence of the regulatory sequences in adjacent loops.

Whatever the nature of the attachment sites, there is strong evidence for the existence of at least one class of insulating element that can serve solely as a domain boundary, with no regulatory activity of its own: the constitutive hypersensitive scs sites (Udvary et al., 1985) found at the ends of the Drosophila 87A7 heat shock locus can insulate reporter genes from the effect of nearby regulatory sequences in transgenic Drosophila (Kellum and Schedl, 1991a, 1991b). Similarly, the Drosophila *gypsy* retrotransposon possesses an insulating activity mediated through the gene *su(Hw)*, though it is not clear whether it plays any role in the formation of natural domain boundaries (Roseman et al., 1993). The A element of the chicken lysozyme domain, which colocalizes with a matrix attachment region (MAR) near the end of the domain, behaves differently from the Drosophila elements, since it has transcriptional activation properties: it shows a moderate insulating effect in transient expression assays but also a strong activating effect in stably transformed cell lines (Stief et al., 1989).

In an attempt to isolate and characterize a "pure" chromatin insulator and potential boundary element in the vertebrate system, we turned to the now well-characterized β -globin domain of chicken, mouse, and human. In all three organisms, important features of the chromatin structure of this domain are well conserved (Figure 1). At the very 5' end, a constitutive hypersensitive site (5'HS5 in human and mouse, 5'HS4 in chicken) is present in all tissue types (Tuan et al., 1985; Forrester et al., 1987; Reitman and Felsenfeld, 1990). Further into the locus, there are erythroid-specific hypersensitive sites that have been demonstrated to function as locus control regions (LCRs) in human and mouse (Grosveld et al., 1987; Forrester et al., 1987, 1989; Ryan et al., 1989; Talbot et al., 1989; Moon and Ley, 1990; Hug et al., 1992; Jimenez et al., 1992b). Studies in transgenic mice and β -thalassemia patients show that the β -globin LCRs are required for decondensation of the higher order chromatin structure of the β -globin domain in erythroid tissues; they serve as potent activators of the expression of all the genes in the β -globin domain. Remarkably, although LCRs decondense the chromatin over more than 200 kb in the 3' direction (Elder et al., 1990), earlier studies suggest that the chromatin near the 5' constitutive hypersensitive site is condensed (Stalder et al., 1980; Kioussis et al., 1983; Reitman and Felsenfeld, 1990).

In light of these observations, we explored the possibility that the 5' constitutive hypersensitive site of the chicken β -globin domain is a domain insulator and therefore blocks the extension of the LCR's influence in the 5' direction. In this report, we demonstrate that the 5' chicken element containing the constitutive hypersensitive site insulates a reporter gene from the influence of a nearby LCR when

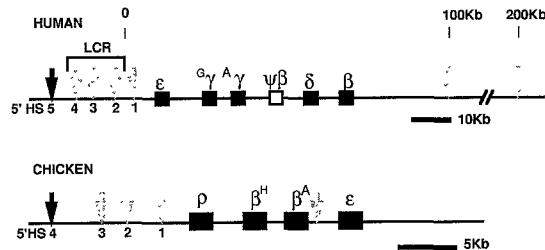


Figure 1. The Map of the Human and Chicken β -Globin Domain
The constitutive hypersensitive sites, 5'HS5 for human and 5'HS4 for chicken, are shown with closed arrows, and the erythroid-specific hypersensitive sites are shown with stippled arrows. The location of the human LCR is also shown.

it is positioned in between them in the human erythroleukemia cell line K562. We show that, when the insulator is in this position, it prevents the establishment of a nucleosome-free region over the promoter of the reporter gene. Furthermore, we find that the insulator, when introduced into *Drosophila*, also serves to protect the *white* minigene from neighboring regulatory elements, suggesting that the insulating function is widely conserved in evolution.

Results

It has been shown that the neomycin (G418) resistance gene driven by the human γ -globin promoter (γ -neo) can be used as a reporter to detect the presence of a globin LCR. When γ -neo is stably introduced into the human erythroleukemia cell line K562, the presence of a mouse or human LCR element (5'HS2) increases the number of G418-resistant colonies 30- to 100-fold (Moon and Ley, 1990). This is consistent with the significant increase in the level of reporter gene transcript that the LCR confers in such erythroid-specific cells. Use of the colony assay is preferable to isolating the transfected cells with a selection marker first and then assaying the level of reporter mRNA or an enzyme activity such as chloramphenicol acetyltransferase, because the colony assay introduces no prior selection bias for integrations into open chromatin.

We employed the colony assay to test whether a 1.2 kb 5' β -globin element containing the chicken constitutive hypersensitive site (5'HS4 in Figure 1) can insulate the γ -neo reporter from a strong LCR (mouse 5'HS2). Constructs in which this chicken 5' element was interposed between the LCR and the γ -neomycin reporter (Figure 2A) were stably transfected into the human erythroleukemia cell line K562. If the chicken 5' element insulates the γ -neo reporter from the LCR, one would expect a significant decrease in the number of G418-resistant colonies. As shown in Figure 2A, presence of one copy of the chicken 5' element on each side of γ -neo (pJC5-4) decreased the number of G418-resistant colonies by 9- to 10-fold as compared with the control, in which the chicken sequence is replaced by the 2.3 kb HindIII-HindIII fragment from λ phage DNA (pJC3-4). Presence of two copies of the chicken 5' element on each side of γ -neo (pJC13-1) de-

creased the number of G418-resistant colonies by approximately 30-fold.

These experiments do not permit us to rule out the possibility that the decrease in the number of G418-resistant colonies is due to an effect of the chicken 5' element on the position of integration or the copy number of the integrated γ -neo reporter construct. To control for such variables, we placed a second selection marker, the herpes simplex virus thymidine kinase (tk)-hygromycin construct, next to the LCR as an internal control (Figure 2B). We stably transfected these constructs into K562 cells and counted the number of G418-resistant colonies and hygromycin-resistant colonies. The number of hygromycin-resistant colonies for all three constructs was similar (data not shown). The ratio of G418- to hygromycin-resistant colonies for each construct varied, as shown in Figure 2B. Consistent with the results shown in Figure 2A, the presence of one copy of the chicken 5' element on each side led to a 7- to 8-fold decrease in this ratio (construct 5-4Neo/

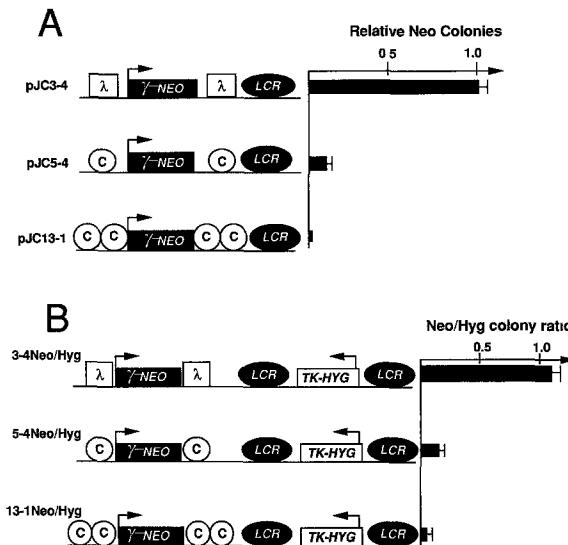


Figure 2. The Chicken 5' Element Insulates the γ -Neomycin (G418) Resistance Gene from the LCR

(A) The human erythroleukemic cell line K562 was stably transfected with the constructs shown on the left and grown in semisolid agar medium containing G418. The G418-resistant colonies were counted after 2 to 3 weeks. The relative numbers of G418-resistant colonies for each construct are shown on the right. The number of colonies for pJC3-4, which contains no chicken 5' element, was arbitrarily set to 1.0. The 1.2 kb fragment containing the chicken constitutive hypersensitive site is marked C. The control fragment, a 2.3 kb HindIII-HindIII fragment from the λ phage DNA, is marked λ . The arrow at the 5' end of the gene labeled NEO indicates the γ -globin promoter of the G418 resistance gene. LCR indicates the location of the mouse 5'HS2. The absolute number of G418-resistant colonies for pJC3-4 in the experiment shown is 268 and 287.

(B) The constructs shown on the left, which include the hygromycin internal control, were transfected and grown in semisolid agar medium containing either hygromycin or G418. The ratio of G418- to hygromycin-resistant colonies for each construct is shown on the right. The arrow at the 5' end of the gene labeled TK-HYG indicates the herpes simplex virus tk promoter of the hygromycin resistance gene. The second LCR was added to enhance the activity of the tk promoter.

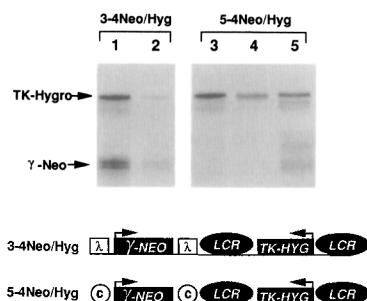


Figure 3. The Chicken Constitutive Hypersensitive Site Insulates the γ -Globin Promoter at the RNA Level
Clonal cell lines containing the constructs 3-4Neo/Hyg (clones 1 and 2) and 5-4Neo/Hyg (clones 3, 4, and 5) from the experiment described in Figure 2B were established by selecting them in hygromycin. The levels of the neomycin resistance gene RNA and the hygromycin resistance gene RNA were analyzed with RNAase protection.

Hyg versus 3-4Neo/Hyg). The presence of two copies on each side led to a 20-fold decrease (construct 13-1Neo/Hyg versus 3-4Neo/Hyg). These findings rule out the possibility that the position of integration or copy number is responsible for the insulating effect seen in Figure 2A. Deleting the LCR from the constructs shown above led to an approximately 40-fold decrease in the number of neomycin-resistant colonies (data not shown). These data show that a single copy of the chicken 5' element, placed between the LCR and the reporter gene, effectively but incompletely insulates the gene (7- to 10-fold instead of 40-fold) from the LCR. However, insulation is nearly complete (20- to 30-fold) when two copies of the chicken 5' element are used. We found that a human constitutive hypersensitive site (5'HS5), located 23 kb upstream of the human ϵ -globin gene, has similar properties. However, it displayed only a 3-fold insulation effect in this assay, probably because there is a moderate enhancer activity residing near the human constitutive hypersensitive site, which partially negates the insulation effect (data not shown).

To determine whether the insulation effect functions at the mRNA level, we characterized clonal cell lines isolated after stable transfection with the two constructs shown in Figure 2B (3-4Neo/Hyg and 5-4Neo/Hyg). These clonal populations were selected with hygromycin, and the mRNA of the neomycin and the hygromycin genes was analyzed by RNAase protection (Figure 3). As expected, when a λ phage DNA spacer was placed between the LCR and the γ -neo reporter (3-4Neo/Hyg), the γ -neo and the tk-hygromycin reporter constructs were expressed at roughly comparable levels in a given clone, although the absolute levels of mRNA varied between clones. By contrast, when one copy of the chicken 5' element was placed between the LCR and the γ -neo reporter (5-4Neo/Hyg), γ -neo reporter expression was either completely absent (clones 3 and 4) or decreased (2-fold, clone 5) relative to the tk-hygromycin reporter. Thus, it appears that insulation with one copy of the chicken 5' element can result in complete suppression of mRNA synthesis but in other cases (e.g.,

clone 5) can be leaky, as expected from the observation that this construct gives rise to a measurable number of G418-resistant colonies (Figure 2B). On Southern blot analysis, the leaky clone (clone 5) has seven copies of the transfected DNA, whereas clones 1 through 4 have only one to three copies. It is possible that when there are numerous copies of the DNA, as in clone 5, some copies escape insulation.

Constitutive Hypersensitive Site Insulates in a Directional Manner

The main operational difference between an insulator and a classic silencer is the directionality of effect. In our assay, the insulator should exert its effect only when it is between the LCR and the γ -neo reporter and not when it is flanking them. In contrast, a classic silencer suppresses gene expression regardless of its position (reviewed by Renkawitz, 1990). To demonstrate directionality, we stably transfected K562 cells with the constructs shown in Figure 4A, in which the γ -neo reporter is flanked by either the chicken 5' element (pJC16) or the control λ phage DNA (pJC17). The data in Figure 4A show that the number of G418-resistant colonies increased slightly when the γ -neo reporter alone was flanked by the chicken 5' element. Similarly, the LCR- γ -neo reporter combination showed a slight increase (less than 2-fold) in the number of G418-resistant colonies when it was flanked by the chicken 5' element (pJC19) rather than by λ phage DNA (pJC20). This suggests that the chicken 5' element does not compete with the γ -neo promoter for the LCR activity; otherwise, flanking the LCR- γ -neo with the constitutive hypersensitive site would decrease the number of G418-resistant colonies. These results show that the chicken 5' element blocks the

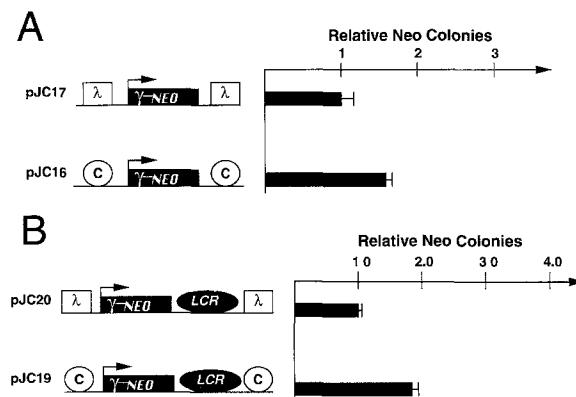


Figure 4 The Chicken and Human Constitutive Hypersensitive Site Insulates in a Directional Manner
(A) The constructs shown on the left were stably transfected, and G418-resistant colonies were counted as described in Figure 2. The relative number of G418-resistant colonies is shown. The number of colonies from the control construct pJC17 was arbitrarily set to 1.0. As in Figure 2, the chicken 5' element is marked C.

(B) The constructs shown on the left were stably transfected and analyzed as in (A). The relative number of G418-resistant colonies is shown. The number of colonies from the control construct pJC20 was arbitrarily set to 1.0.

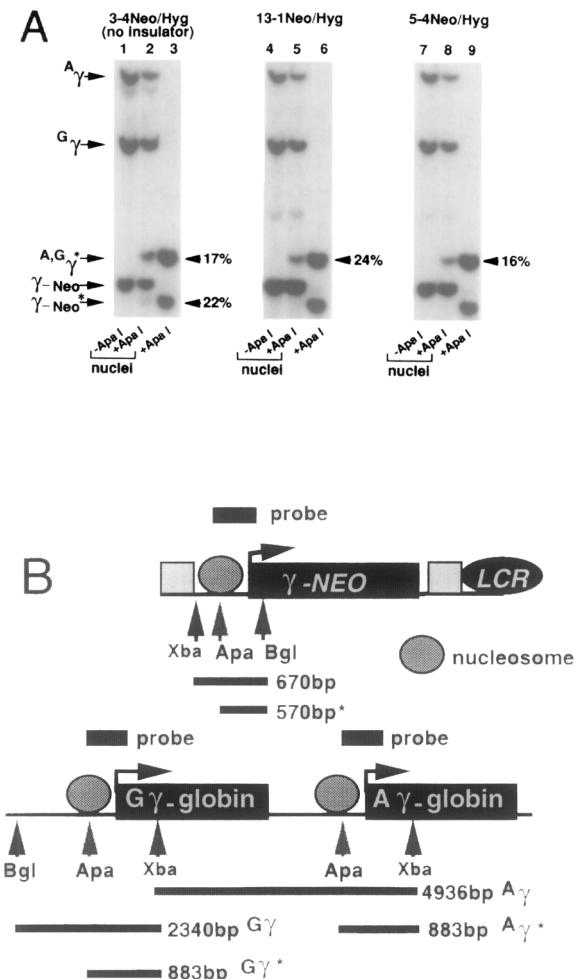


Figure 5. The Chicken Chromatin Insulator Blocks the LCR from Displacing the Nucleosome over the γ -Neomycin Promoter

(A) Nuclei of pooled clones (100 to 200 clones) selected with hygromycin from the transfection shown in Figure 2B were either mock digested (lanes 1, 4, and 7) or digested with Apal (lanes 2, 5, and 8) before genomic DNA was isolated. The bands generated by Apal cutting in nuclei are marked ${}^{\text{A}}\text{G}^{\text{A}}\gamma^*$ and $\gamma\text{-Neo}^*$ for the endogenous ${}^{\text{A}}\gamma$ - and ${}^{\text{G}}\gamma$ -globin promoters and γ -neomycin promoter, respectively (see [B] for details). In lanes 3, 6, and 9, the genomic DNA was isolated prior to being digested to completion with Apal. The percentage of cutting at each Apal site as determined by a PhosphorImager from this Southern blot is shown on the right for each construct. The percentage was determined by dividing the intensity of the bands resulting from the Apal digestion in lanes 2, 5, and 8 by that of the corresponding bands in lanes 3, 6, and 9, respectively. The genomic DNA for all nine lanes was digested with BgIII and XbaI to generate the parental bands.

(B) The maps of the transfected DNA as well as the endogenous γ -globin genes are shown. The squares flanking the γ -neomycin gene indicate the location of either the control DNA (in 3-4Neo/Hyg) or the chromatin insulator (in 13-1Neo/Hyg and 5-4Neo/Hyg). The tk-hygromycin gene that is linked to the γ -neomycin gene is not shown here (see Figure 2B). The probe, which is derived from the ${}^{\text{A}}\gamma$ -globin promoter, is shown along with the expected fragments in the Southern blot shown in (A).

effect of the LCR only when it is placed between the LCR and the reporter gene. Since it does not have a significant regulatory activity of its own either as a silencer or an enhancer, it must be considered to have only the function

of an insulator. The slight increase in the number of G418-resistant colonies seen when the 5' element flanks the LCR- γ -neo reporter combination (Figure 4B) might result from the 5' element preventing other nearby promoters from competing with the γ -neo promoter for the LCR activity. From here on, we shall refer to the chicken 5' element as an insulator.

Insulator Blocks the LCR from Displacing the Nucleosome over the Promoter

Data from naturally occurring β -thalassemias, transgenic mice, and transfection studies indicate that the LCR plays a role in creating and maintaining a region either free of nucleosomes or with an altered nucleosome structure over the promoters of the genes in the β -globin domain and in forming an active transcriptional complex (reviewed by Felsenfeld, 1992). By examining the accessibility of a restriction enzyme site in the promoter of the reporter gene, we can ask whether the presence of the insulator affects chromatin structure at the promoter.

In the Southern blot shown in Figure 5, we tested for the presence of an active chromatin configuration at the γ -neo promoter by examining the accessibility of the Apal restriction enzyme site in the γ -neo promoter. Intact nuclei of K562 cells, transfected with the constructs shown in Figure 2B, were digested with Apal, and the genomic DNA was isolated and cut with XbaI and BgIII to generate the parental fragments. If chromatin structure over the γ -globin gene promoter is undisrupted, as in HeLa cells where the γ -globin promoters and the LCRs are inactive, the Apal site is inaccessible (E. Bresnick, personal communication). If a nucleosome at the promoter is displaced or disrupted, the Apal site is accessible to Apal and is cut at that site. This generates 570 bp and 883 bp fragments (marked with asterisks) from the γ -neo and the endogenous γ -globin promoters, respectively, which are detected when probed with the ${}^{\text{A}}\gamma$ promoter, as shown in Figure 5B. In K562 cells, which express the ${}^{\text{A}}\gamma$ and ${}^{\text{G}}\gamma$ -globin genes, the nucleosomes over these promoters are displaced (E. Bresnick, personal communication). Because the promoter of the γ -neo reporter is derived from the endogenous ${}^{\text{A}}\gamma$ -globin gene and the promoters of the endogenous ${}^{\text{A}}\gamma$ and ${}^{\text{G}}\gamma$ -globin genes are identical, we can use the same Southern blot to compare the accessibility of the Apal site in the γ -neo reporter with that in the two endogenous γ -globin gene promoters.

As shown in Figure 5A, the Apal site in the endogenous γ -globin promoters (${}^{\text{A}}\text{G}^{\text{A}}\gamma^*$) was cut with 16%–24% efficiency in each of the three constructs. Similarly, the Apal site in the γ -neo promoter ($\gamma\text{-Neo}^*$) was cut with about the same efficiency, and the parental γ -neo band correspondingly decreased in construct 3-4Neo/Hyg (22%), which has no insulator (lane 2). However, cutting at γ -neo promoter ($\gamma\text{-Neo}^*$) was not visible when the LCR was blocked by the insulator (lanes 5 and 8) in constructs 13-1Neo/Hyg and 5-4Neo/Hyg. We conclude from this experiment that the nucleosome over the γ -neo promoter is intact when the LCR is blocked by the insulator (13-1Neo/Hyg and 5-4Neo/Hyg) but is displaced or disrupted when the LCR is not blocked (3-4Neo/Hyg).

Chromatin Insulator Confers Position Independence in Transgenic Drosophila

We have demonstrated that the chicken element functions as an insulator in the human erythroid cell line K562. Can it function in even more divergent species and with other regulatory elements? We addressed this question by employing P element-mediated transformation (reviewed by Wilson et al., 1990) to introduce plasmid constructs containing the *white* minigene flanked with two copies of our insulator sequences. If these sequences act as insulators in Drosophila, the expression of the *white* minigene should be protected against position effects in independent insert fly lines (Kellum and Schedl, 1991a; Roseman et al., 1993). The Drosophila *white* minigene is particularly convenient, as its expression in the eye provides a sensitive and easily scored assay. The level of the *white* minigene expression directly affects eye color: low levels of expression give a pale yellow eye, while high levels result in a red eye. In the absence of insulating elements, flies transformed with the *white* minigene display a range of eye colors varying from white to red, depending on the level of expression of the gene, which is in turn dependent on the nature of the regulatory elements and chromatin structure near the site of integration (Hazelrigg et al., 1984; Levis et al., 1985; Pirrotta et al., 1985). Based on studies using Drosophila *scs* and *gypsy* retrotransposon, we would expect that, if the insulator is able to function in Drosophila, the eye color in these lines should not vary with the site of integration and, since the *white* minigene is normally expressed at a low level, should be pale yellow.

We developed a total of four independent lines containing a single copy of the construct pJC99, in which the *white* minigene is flanked by the chicken insulator, and three lines containing a single copy of the control construct pJC100, in which the *white* minigene is flanked by the λ phage DNA (Figures 6A and 6B). For all lines, a strain of Drosophila with inactive endogenous *white* genes, and therefore with white eye color, was used. All four insulated lines had pale yellow eyes (Figure 6A), whereas the three uninsulated lines had eye colors ranging from pale yellow to orange (two representative lines are shown in Figure 6B). Therefore, flanking the *white* minigene with the chicken insulator protects against activating elements near the integration site in Drosophila in a way similar to the Drosophila sequence element *scs* (Kellum and Schedl, 1991a) and the Drosophila retrotransposon *gypsy* (Roseman et al., 1993).

In eye color assays, homozygotes for the introduced *white* minigene typically have noticeably darker eye colors than heterozygotes, because there are two identical copies of the *white* minigene instead of one (Pirrotta, 1988). In the course of this study, we observed an unexpected phenomenon (Figure 6C). Among the four lines containing the chromatin insulator that we have tested (lines 99-1, 99-2, 99-3, and 99-4), line 99-2 had a darker eye color in a homozygote than in a heterozygote, as one would expect, but lines 99-3 and 99-4 had the same eye color in homozygotes and heterozygotes (only lines 99-2 and 99-4 are shown in Figure 6C). This was consistently reproducible in separate homozygote-generating crosses. Line

99-1 is homozygous lethal; hence, the difference between heterozygotes and homozygotes could not be observed. This observation suggests that in lines 99-3 and 99-4, where the *white* minigene is flanked by the insulator, one *white* minigene is able to sense the presence of the other *white* minigene on the homologous chromosome, and as a result one of the two alleles is turned off, or both are partially suppressed. Such pairing effect has been seen with the *zeste* binding sites (reviewed by Wu and Goldberg, 1989) and *enrailed* regulatory region (Kassis et al., 1991).

Discussion

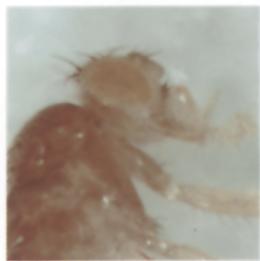
There is considerable evidence that, within the eukaryotic nucleus, chromatin components serve to divide the genome into structurally and functionally independent domains (reviewed by Eissenberg and Elgin, 1991). The presence of domains implies that there exist boundaries that define their ends. To understand how the higher order chromatin structure of a domain is regulated as a unit, it is crucial to understand how such boundaries are formed. Of particular interest are the domains containing transcriptionally active genes, which display a relatively uniform and increased level of sensitivity to DNAase I often extending over many kilobases on either side of their coding regions. Boundaries to such a domain may serve to confine enhancers and locus control regions to act only within the domain, as well as to insulate the genes within from the *cis*-acting regulatory elements or chromatin structure of an adjacent domain.

In this report, we have characterized an element at the 5' end of the chicken β -globin locus that appears to serve as a pure insulator without significant regulatory activity of its own. The chicken globin insulator thus behaves like the *scs* element of Drosophila (see below) but is distinctly different in character from other elements, such as an LCR, that confer position independence. The individual hypersensitive sites (5'HS2-4) that compose the LCR of the human β -globin domain, when integrated into the mouse genome, appear to confer both position independence and a high level of expression. Since these sites work in concert, individual sites clearly do not insulate the promoter from the effects of sites further upstream. In these cases, it is likely that a strong interaction between the LCR and the reporter gene makes it oblivious to its surroundings and, therefore, position independent in expression. A similar situation appears to exist in the case of the chicken lysozyme domain A element (Stief et al., 1989). Although this may contain an insulating activity, it also strongly activates transcription from nearby promoters when stably integrated in cell lines.

MARs such as the A element have also been postulated to form domain boundaries (Stief et al., 1989). Clearly, not all MARs form domain boundaries, since some of them reside within genes (Cockerill and Garrard, 1986). The chromatin insulator that we have described here does not appear to have *in vitro* MAR activity (data not shown). However, we cannot rule out the possibility that the insulator is indirectly associated with the nuclear matrix or interacts with MARs within the nucleus.

A

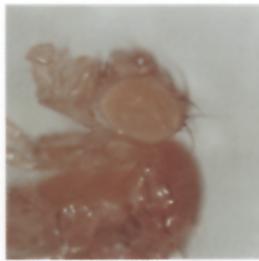
99-1



99-2



99-3



99-4



B

100-1



100-2



C

99-2



heterozygote

homozygote

99-4



heterozygote

homozygote

Figure 6. The Chromatin Insulator Protects the *white* Minigene from Position Effect in Drosophila

The constructs shown in (A) and (B) were injected into a Drosophila strain with inactive endogenous *white* genes and, therefore, white eye color.
(A) Eye color phenotype of the four lines containing pJC99, in which the *white* minigene is flanked by the insulator.

(B) Eye color phenotype of two representative lines containing the control construct pJC100, in which the *white* minigene is flanked by the λ fragment.

(C) Eye color phenotype of lines 99-2 and 99-4 in heterozygous state and in homozygous state.

As far as we know, there is only one insulator at the 5' boundary of the chicken β -globin domain, and yet we find that the presence of only one insulator on each side of the reporter gene (constructs pJC5-4 and 5-4Neo/Hyg, Figure 2) reduces the effect of the LCR only 8- to 10-fold (instead of full 40-fold). Two copies of the chromatin insulator are required on each side (constructs pJC13-1 and 13-1Neo/Hyg) to block the LCR completely. There are several possible explanations of this result: First, there might be elements in the 5' region other than the one we have studied that are required for full insulator activity. Second, the 5' insulator might have to interact with a complementary chromatin insulator, yet to be identified, at the 3' boundary of the β -globin domain for a full insulator activity. Third, the distances between the LCR, the insulator, and the reporter gene promoter might be critical for *in vivo* activity, and these distances might be too close in the transfected DNA constructions. Fourth, during the integration of the transfected DNA, the LCR-promoter complex could in some cases have formed before the insulator complex had a chance to form. It appears, however, that whatever is lacking when we use one copy of the chromatin insulator is compensated for by having two copies, which provide almost complete insulation.

The fact that the insulator prevents position effects in Drosophila eye cells shows that the insulator is capable of blocking the effects of a wide variety of regulatory elements and perhaps chromatin structures, that its effect is not confined to erythroid cells, and that it can function across a wide evolutionary spectrum. The fact that the activity is evolutionarily conserved strongly suggests the importance of its role in chromatin domain organization and implies that similar chromatin insulators are present in the boundaries of other domains in other organisms.

The insulator appears to be able to mediate pairing effect in Drosophila (reviewed by Tartof and Henikoff, 1991), perhaps by facilitating physical interaction between the homologous alleles. Similar interaction in trans has been seen with other elements that mediate pairing effect, such as the *zeste* binding sites (reviewed by Wu and Goldberg, 1989) and the *engrailed* regulatory region (Kassis et al., 1991). The pairing effect of the insulator might be conserved in vertebrates as well. If the insulators are distributed throughout the length of the chromosome, they might facilitate homologous chromosome pairing and sister chromatid exchange during meiosis.

How does the chromatin insulator block the influence of a nearby regulatory element, such as a strong enhancer or LCR? We know from studies of the naturally occurring β -thalassemias, as well as transgenic mice, that the LCRs play a critical role in displacing the nucleosome over the promoter of the genes in the β -globin domain and in forming an active transcriptional complex. Our findings suggest that the chromatin insulator prevents the LCR from disrupting the nucleosome at the promoter and presumably blocks the formation of a transcriptional complex at the promoter as well. Exactly how the chromatin insulator does this is not clear, but three models can be proposed (Figure 7). In model A, the LCR and its bound factors track along the DNA, looping out the intervening sequences in

search of the target promoter. In this model, the chromatin insulator blocks the LCR complex from reaching the target promoter. In model B, the LCR complex searches for the target promoter not by tracking but by skipping along the DNA, again looping out the intervening sequences (Müller et al., 1989). The insulator, in this model, somehow reduces the accessibility of the promoter to the LCR complex, perhaps by immobilizing the intervening DNA on a nuclear membrane or matrix. In model C, the 5' chromatin insulator forms a complex with the 3' chromatin insulator to generate a loop that excludes the LCR, blocking the LCR by immobilizing the intervening sequence or topologically isolating the LCR (Udvary et al., 1985). More information is needed before any of these models can be ruled out. This information may be more difficult to obtain for the insulator than for other kinds of *cis*-regulatory elements, since preliminary experiments (data not shown) on the effect of deletions within the insulator indicate that DNA sequences contributing to the insulating activity may be distributed over much of its 1.2 kb length.

The ability of the chicken 5' element to function in human cells and to behave as an insulator without accompanying activation or silencing effects within the protected domain may have valuable practical implications. Present gene therapeutic techniques introduce genes randomly into the chromatin environment, where they may act on or be affected by local chromatin structure and regulatory elements. Such genes may experience immediate or gradual loss of expression, perhaps due to the repressive influence of the surrounding sequence (Palmer et al., 1991; Scharfmann et al., 1991). It is also possible for the transfected gene to be integrated near an oncogene, which may be activated by the *cis*-acting elements of the transfected gene. The availability of an insulating element should be helpful in reducing these unwanted side effects, as well as in other experiments with transgenic animals. As the data presented above make clear, this element also makes possible experiments with stably transformed cell lines in which an introduced gene is insulated from the effects of an adjacent selectable marker.

Our results add to the growing body of evidence that certain chromatin elements serve *in vivo* to block directionally the interactions between activators (enhancers or LCRs) and promoters. The insulator we describe appears

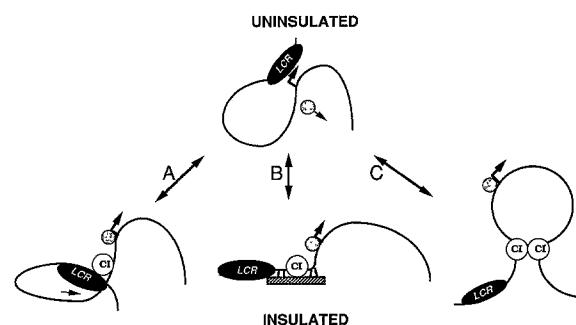


Figure 7. Models of Insulation

Three models of insulation are shown, as described in Discussion.

to be conserved through evolution, and therefore it seems likely that similar elements play a role in boundary formation in a wide variety of gene clusters and organisms.

Experimental Procedures

Construction of Transfection DNA

The plasmid constructs shown in Figure 2A were constructed as follows: The plasmids pJC3-4, pJC5-4, and pJC13-1 were constructed by initially inserting the 1.1 kb EcoRI-EcoRI fragment (Moon and Ley, 1990) containing the mouse 5'HS2 (LCR) into the EcoRI site of the vector pGEM-4Z (Promega). The 2.7 kb BamHI-BamHI fragment containing the human γ -globin promoter linked to the neomycin (G418) resistance gene (Moon and Ley, 1990) was then inserted into the BamHI site of this plasmid. To make pJC3-4, the 2.3 kb HindIII-HindIII fragment from the λ phage DNA was blunted with Klenow and cloned into the blunted SacI site and XbaI site using an XbaI linker. The plasmid pJC5-4 was made similarly by inserting the 1.2 kb SacI-SspI fragment from pCBGC (Reitman and Felsenfeld, 1990) containing the chicken constitutive hypersensitive site (5'HS4) into the SacI and XbaI sites after ligating the corresponding linkers. The plasmid pJC13-1 was made by inserting one 1.2 kb SacI-SspI (chicken 5'HS4) fragment in the KpnI site, one in the SacI site, and two in the XbaI site. To create the constructs shown in Figure 2B, we first created plasmid pJC78 by inserting the herpes simplex virus tk-hygromycin resistance gene from pHg (Sugden et al., 1985) into the SacI-BamHI site and the mouse 5'HS (LCR) into the EcoRI site of vector pGEM-4Z. The Asel-XmnI fragment containing the 5'HS2 and tk-hygromycin was isolated from pJC78 and ligated to plasmids pJC3-4, pJC5-4, and pJC13-1, which had been cut with NdeI and SalI, to create 3-4Neo/Hyg, 5-4Neo/Hyg, and 13-1Neo/Hyg, respectively. The ligated product was isolated from low melting agarose gel.

The plasmid pJC16 was made by removing the EcoRI-EcoRI fragment containing the mouse 5'HS2 from pJC5-4. The plasmid p17 was constructed by replacing the SacI-SacI and XbaI-XbaI inserts of pJC16 with a 950 bp EcoRI-HindIII fragment from the λ phage DNA ligated to either SacI or XbaI linkers, respectively, after blunting with Klenow. Plasmids pJC19 and pJC20 were made by inserting the 1.1 kb fragment containing the mouse 5'HS2 into the KpnI site of pJC16 and pJC17, respectively, after a KpnI linker was ligated.

To create pJC99, the white minigene was excised with EcoRI from pCasper W15 (gift of V. Pirrotta), blunted with Klenow, and ligated into the BamHI site of pJC13-1, using a BamHI linker and thereby replacing the γ -neomycin gene. pCasper W15 is identical to pCasper (Pirrotta, 1988) except that it has an EcoRI site on both sides of the white minigene. The resulting plasmid was then cut with SphI, blunted with Klenow, and ligated to Spel linkers. After digestion with Spel, an Spel-Spel fragment containing the white minigene flanked by the chromatin insulator was cloned back into pCasper W15 whose EcoRI sites were changed to Spel sites using Spel linkers. To create pJC100, we first created pJC14-4, which was made by inserting the 1.1 kb EcoRI-EcoRI fragment containing the mouse 5'HS2 into the EcoRI site of pJC16 (see above). The white minigene was excised with EcoRI from pCasper W15, blunted with Klenow, and ligated into the BamHI site of pJC14-4 using a BamHI linker, thereby replacing the γ -neomycin gene. The resulting plasmid was then cut with SphI, blunted with Klenow, and ligated to Spel linkers. After digestion with Spel, a Spel-Spel fragment containing the white minigene flanked by the λ DNA was cloned back into pCasper W15 that had its EcoRI sites changed to Spel sites using Spel linkers.

K562 Cell Transfection and Colony Assay

In a typical experiment, 10^7 mid-log phase K562 erythroleukemia cells (Ney et al., 1990) were harvested and washed once with phosphate-buffered saline before resuspension in 0.5 ml of cold phosphate-buffered saline. The linearized DNA (0.25 μ g) was added and mixed. After 10 min on ice, the cells were electroshocked using the Bio-Rad Gene Pulser at 200 V and 960 μ F. After 15 min on ice, the transfected cells were transferred to 35 ml of improved minimal essential medium containing 10% fetal calf serum. For constructs pJC16 and pJC17, 10 μ g of linearized DNA was used. To generate neomycin (G418)- or hygromycin-resistant colonies, 3 ml of transfected cells was diluted

1:10 in improved minimal essential medium containing 10% fetal calf serum, 0.3% cell culture agar (Sigma), and 500–1000 μ g/ml of active G418 (GIBCO) or 300 U/ml of hygromycin (Sigma), respectively, and plated in petri dishes 1 to 2 days after transfection. The G418- and hygromycin-resistant colonies were counted 2 to 3 weeks after selection.

RNAase Protection Assay

Clonal cells from the experiment shown in Figure 2B were isolated and grown in hygromycin. After the copy number of each clone was determined by a Southern blot, RNA was isolated from four clones with RNAzol (Cinna/Biotecx). RNAase protection was performed on 30 μ g of RNA with the RPA II kit (Ambion). The probe for the γ -neo reporter RNA was derived from a 457 bp BamHI-AlwNI fragment in plasmid γ -neo (Moon and Ley, 1990) containing the γ -globin promoter, which was then cloned into the BamHI-AlwNI site of pBluescript II SK(+) (Stratagene). The RNA probe was synthesized with the mRNA capping kit (Stratagene) in the presence of [α - 32 P]UTP and T7 RNA polymerase. The probe protects a 143 bp band specific for the γ -neo RNA. The probe for tk-hygromycin reporter RNA was derived from the 333 bp MluI-EcoRI fragment in pHg (Sugden et al., 1985), which was cloned into the SmaI-EcoRI site of pBluescript II SK(+) and linearized at the SmaI site within the hygromycin gene. The RNA probe was synthesized with the mRNA capping kit (Stratagene) in the presence of [α - 32 P]UTP and T3 RNA polymerase. The probe protects a 260 bp band specific for the tk-hygromycin RNA.

Nuclei Digest with Apal Restriction Enzyme

Pooled cells representing 100 to 200 clones from the experiment shown in Figure 2B were grown in hygromycin. Approximately 3×10^7 cells were spun down and washed once with cold phosphate-buffered saline. The cells were then resuspended in 400 μ l of lysis buffer containing 10 mM Tris-HCl (pH 7.5), 10 mM NaCl, 3 mM MgCl₂, 0.2% Nonidet P-40, and 5 mM dithiothreitol and immediately spun in a Tomy MTX150 microcentrifuge for 4 min. The nuclear pellet was then washed once with a wash buffer containing 10 mM Tris-HCl (pH 7.5), 10 mM NaCl, 3 mM MgCl₂, and 5 mM dithiothreitol by spinning in Tomy MTX150 microcentrifuge for 2 min. The nuclear pellet was resuspended in 400 μ l of digestion buffer containing 100 U of Apal, 50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, and 1 mM dithiothreitol and incubated in 30°C for 20 min. The genomic DNA was isolated by the standard methods and digested to completion with BglII and XbaI. The digested DNA (15 μ g) was analyzed with the standard Southern blot method and probed with a 335 bp BamHI-HindIII fragment from the plasmid γ -Neo (Moon and Ley, 1990) containing the γ -globin promoter. The probe was labeled with [α - 32 P]dCTP using the random primed DNA labeling kit (Boehringer Mannheim). The hybridization was performed in QuikHyb rapid hybridization solution (Stratagene).

Generation of Transgenic Drosophila Lines

Injections were done by standard procedures (Spradling, 1985) into a homozygous *Df(1)w67c2, y* strain. Some of the lines used in this study were generated by mobilization of P element constructs by crossing established lines to a strain containing P element transposase (Robertson et al., 1988). The chromosomal assignments of insertions were determined by segregation tests, using balancer stocks for the second and the third chromosome (second chromosome balancer stock: *wDp(2;Y)A161, B⁺;nub b Sco It stw³SM6a*. Third chromosome balancer stock: *y w;TM3, y⁺ ri⁰ sep bx 34^o e^o Ser/Sb*). Inverse polymerase chain reaction was used to determine that all lines used contained a single P element insert. This was done by cutting the genomic DNA with either HhaI or DpnII and using 3' P element primers as described by Whiteley et al. (1992).

Photography

Heterozygous female flies were photographed under a Nikon dissecting microscope using Kodachrome 40 type film. Flies were collected shortly after hatching and then were aged overnight before taking the photographs.

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