Positional enhancer-blocking activity of the chicken β-globin insulator in transiently transfected cells

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It is thought that insulators demarcate transcriptionally and structurally independent chromatin domains. Insulators are detected by their ability to block enhancer–promoter interactions in a directional manner, and protect a transgene from position effects. Most studies are performed in stably transformed cells or organisms. Here we analyze the enhancer-blocking activity of the chicken β-globin insulator in transient transfection experiments in both erythroid and nonerythroid cell lines. We show that four tandem copies of a 90-bp fragment of this insulator were able to block an enhancer in these experiments. In circular plasmids, placement on either side of the enhancer reduced activity, but when the plasmid was linearized, the enhancer-blocking activity was observed only when the insulator was placed between the promoter and the enhancer. These observations are consistent with the position-dependent enhancer-blocking activity of the insulator observed in stable transformation experiments.

Eukaryotic nuclear and genomic organization contributes to the transcriptional regulation of tissue- and stage-specific genes or groups of genes. In particular, it has been proposed that the organization of chromatin into defined domains helps to facilitate and maintain gene expression. This raises the questions of how an active domain could be established and its extent determined. The identification of DNA sequences that appear to play a role as boundary elements has provided a focus for addressing these questions. The specialized chromatin structures (*scs* and *scs′*) from the *Drosophila* 87A7 heat-shock locus were the first examples of boundary elements (1). Functionally, the *scs′* element was identified as a chromatin boundary through its ability to confer position-independent expression on a reporter gene which determines eye color (2). In subsequent experiments, *scs′* has been shown also to possess position-dependent enhancer-blocking activity in *Drosophila* (3).

Other studies identified the *Drosophila gypsy* retrotransposon as another enhancer-blocking element. The enhancer-blocking activity of *gypsy* depends on the presence of multiple binding sites for the product of the gene *suppressor of Hairy-wing* [*su(Hw)*] (4). In experiments analogous to those with the *scs* elements described above, it has been shown that a reporter gene surrounded by these sites is protected against position effects (5).

The early experiments with these *Drosophila* elements were performed with transformed organisms. Subsequent studies with both *scs′* and *gypsy* have also detected enhancer-blocking activity in stably transformed *Drosophila* cell lines. Mixed results have been obtained in assaying insulator activity in a nonintegrated context. It has been shown that *gypsy* sites block enhancers in transient expression assays in *Drosophila* cell lines (6). In contrast, the *scs′* element is inactive in such an assay (7); however, in a variant of this experiment, *scs* and *scs′* were shown to function in an enhancer-blocking assay when the reporter constructs were microinjected as plasmids into *Xenopus laevis* oocytes, demonstrating that these insulator elements do not necessarily require integration into the genome to exert their blocking activity (8, 9). Similar results have been obtained in transient expression studies with the A-elements that demarcate the 5′ and 3′ boundaries of the chicken lysozyme gene (10). In addition to conferring position-independent transgene expression, the A-element functions in transient transfections to block enhancer–promoter interactions only when it is between, but not when it is outside, the enhancer and the reporter (10). These results suggest that boundary elements can maintain the enhancer-blocking function in integrated and nonintegrated environments (6, 10).

Earlier studies have shown that multiple copies of a 250-bp "core" element of the chicken 5′hypersensitive site 4 (HS4) have strong insulator activity as measured by an enhancer-blocking assay in stably transformed human erythroleukemic K562 cells (Fig. 1) (11–13). Recently, we were able to identify a 90-bp DNA sequence, within the 250-bp core fragment, which also possesses significant enhancer-blocking activity when present in multiple copies, as measured by the same assay (Fig. 1) (15). *In vitro* DNase I footprint experiments that used nuclear extracts from human K562 and HeLa cells showed the presence of two binding sites for nuclear factors in this 90-bp DNA fragment, and by sequence analysis, three potential Sp1-binding sites (13, 15). When the wild-type 90-bp fragment is multimerized, the blocking activity, in the integrated context, increases proportionally to levels even higher than those observed with the original 1.2-kb fragment (15). Because the mechanism of this enhancer-blocking activity is poorly understood, it is important to devise a simple system in which it can be studied. Here we have measured the effects of smaller fragments of the 1.2-kb chicken β-globin insulator in transient transfection experiments, i.e., in a nonintegrated context. We have examined insulator action by inserting these elements at different locations into circular plasmids, and transiently transfecting both nonerythroid and erythroid cells. We find that the presence of the insulator elements has a major effect on the expression of the reporter gene. On circular templates, decreased expression is observed wherever the insulator is situated with respect to the enhancer; however, when the plasmids are linearized before transient transfection, decreased activity is observed only when the insulator element is placed between the enhancer and the promoter. This result is consistent with the position-dependent enhancer-blocking activity of insulators observed in stable transformation experiments; it places a number of restrictions on possible mechanisms of action of the insulator and also suggests mechanisms of enhancer action.

Materials and Methods

Plasmids and Constructs. The chicken (II/III-Ins)Q fragment (Fig. 1) was liberated from pNI-II/IIIQ by digestion with *Bam*HI and *Eco*RI and cloned in the *Xho*I site of the pGL2-control vector (Promega) by using *Xho*I-linkers (New England Biolabs). The 1.2-kb and the core insulator fragments were cloned in a new *Sal*I

Abbreviations: SV40, simian virus 40; CAT, chloramphenicol acetyltransferase.

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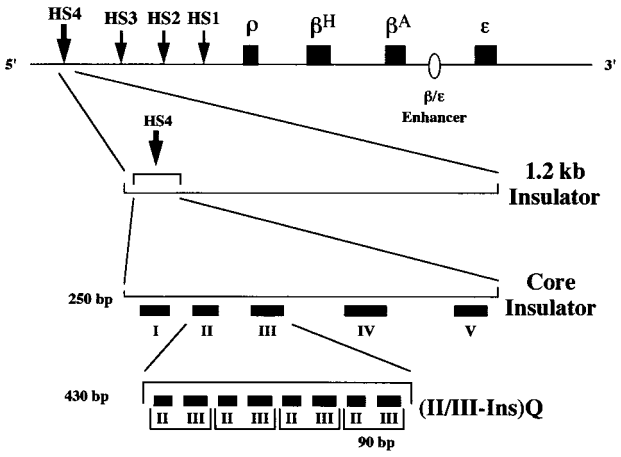

Fig. 1. The chicken β-globin gene cluster. The diagram on top shows the positions of the globin genes and the β/ε enhancer; the erythroid-specific hypersensitive sites (HS1, 2, and 3) and the constitutive 5′HS4 site are shown with arrows. The original 1.2-kb chicken β-globin insulator and the core insulator with fine mapping of the hypersensitive site are schematically shown underneath. The black boxes represent DNA-binding sites for nuclear factors; (II/III-Ins)Q is a DNA fragment composed of four copies of a minimal element (15), a 90-bp DNA fragment that includes the DNA-binding sequences FII and FIII defined previously (15). The four copies of the 90-bp fragment are organized head-to-tail to form a 430-bp fragment.

site introduced in the polylinker of the pGL2-control vector. For the pGL2-enhancer, pGL2-promoter and pGL2-basic series, the (II/III-Ins)Q was cloned in the *Xho*I site. Where the insulator flanked the simian virus 40 (SV40) enhancer in the pGL3 series of vectors, it was cloned into a *Bam*HI and a new *Pst*I site located between the luciferase poly(A) site and the SV40 enhancer. The *Pst*I site was introduced by site-directed mutagenesis into the pGL3 vector (Promega) by using the QuickChange mutation kit (Stratagene). The primers used for the introduction of the *Pst*I site were: up, 5′-CTACAAATGTGGTAAACTGCAGATCG-ATTAAGGATCTGAAC-3′ and down, 5′-GTTCAGATCCT-TATCGATCTGCAGTTTACCACATTTGTAG-3′. For the chicken β-globin promoter–enhancer, we used chloramphenicol acetyltransferase (CAT) reporter vectors under the control of the chicken adult β^A-globin promoter and the β/ε enhancer, described as the 120-bp "EP" fragment (11). The enhancer-less vector was constructed by deleting the enhancer with *Sal*I-*Hin*dIII restriction enzyme digestion and religation. Various insulator fragments were cloned in the *Sal*I and/or the *Hin*dIII site by using the respective linkers. The 90-bp insulator and the same fragment with Sp1 site mutations were obtained by *Asc*I digestion from the pNI-(2/3S) and pNI-(2/3S-ΔSp1) plasmids (15). The mutated 90-bp fragment (*ctt* mutation defined in ref. 15) was synthesized with a *Sal*I site on each end. The deleted and mutated 90-bp fragments were filled in and a *Sal*I-linker was ligated. All of these fragments were cloned in the *Sal*I site located between the chicken β^A-globin promoter and the β/ε enhancer. The influence of the orientation of the (II/III-Ins)Q was not systematically examined in these studies. In the pGL3 series (Fig. 3*A*), the (II/III-Ins)Q fragment cloned into the *Bam*HI site between the enhancer and the promoter (construct 3c) is oriented head-to-tail in the same direction as transcription, whereas the (II/III-Ins)Q fragment cloned into the *Pst*I site upstream of the enhancer is oriented opposite to the direction of transcription (construct 3f). In the β/ε series (Figs. 3*B* and 5), the (II/III-Ins)Q fragment cloned into the *Sal*I site between the enhancer and the promoter (constructs 3h and 5b) is oriented in the same orientation as transcription whereas this fragment is in the opposite orientation in the *Hin*dIII site upstream of the enhancer (constructs 3j and 5c).

Cell Culture and Transfections. 6C2 is a cell line generated by transformation of chicken bone marrow with a wild-type avian erythroblastosis virus and provides a source of cells arrested at the CFU-E stage (16). 6C2 cells were grown in minimal essential medium (α-MEM; GIBCO/BRL) supplemented with 10% FCS, 2% chicken serum, 1 mM Hepes, pH 7.2, 50 μM 2-mercaptoethanol, 100 units of penicillin and 0.1 mg of streptomycin per ml at a density of 1–2 × 10^7/ml. For plasmid transfection, cells were washed twice in ice-cold PBS, resuspended in 0.5 ml of PBS, and mixed with DNA in an electroporation cuvette. After allowing the cells and DNA to stand on ice for 10 min., they were shocked by using a Bio-Rad Gene Pulser at 200 V, 950 μF. After a 15-min recovery on ice, cells were transferred to a 100-mm dish with fresh medium; 48 h later, the cells were harvested, washed twice with cold PBS, and lysed in 200–400 μl of Reporter Lysis Buffer (Promega). Cell extract (20 μl) was added to 50 μl of Luciferase Assay Substrate (Promega) at room temperature and assayed for luciferase activity in a Berthold Lumat LB9501 luminometer. Measured luciferase activity was normalized to the β-galactosidase activity from the pTKβ reporter plasmid (CLONTECH). The assay background was determined with cells electroporated in the absence of DNA. For the QT6 quail embryo fibroblast transformed cell line, the transfections were performed by using 1–2 × 10^7 cells per sample (17). Cells were washed twice in PBS and resuspended in up to 3 ml of Opti-MEM (GIBCO/BRL) containing 5 μl of LipofectAMINE (GIBCO/BRL) and DNA at a concentration which gave a 1:1 DNA:liposomes ratio. Test plasmid (5–10 μg) per sample was slowly mixed with the liposomes and incubated at room temperature for 15 min before adding Opti-MEM; cells were incubated at 37°C for 5–6 h in the presence of transfection mix, returned to normal media, and incubated for 48 h.

Transient Transfection in 10-Day-Old Erythrocytes. Transient transfection in erythroid cells from 10-day-old embryos was performed by electroporation (18, 19). Cells (10^8 in 0.5 ml of L-15 medium) were mixed with 5 μg of RSV-luciferase plasmid and 10 μg of test plasmid. After 5 min at room temperature, cells were electroporated (0.4 cm cuvette, 500 μF, 450 V, for 9 ms) by using a Bio-Rad gene pulser. Cells were cultured with 1.4 ml of complete L-15 medium at 37°C for 24–48 h.

Transfections with Linearized Plasmids. For linearized plasmid transfections, the RSV-luciferase and chicken β^A-globin promoter-insulator-β/ε enhancer series of plasmids were linearized at the *Xmn*I restriction site located in the ampicillin resistance gene. This digestion leaves at least 1.9 kb of plasmid DNA on both sides of the test constructs. The linearized plasmids were gel purified and quantified by UV absorbance. For each transfection, 10^8 ten-day-old chicken erythrocytes were electroporated in 0.5 ml of L-15 medium with 5 μg of the test plasmids and the normalizing plasmids. After electroporation, 1 ml of complete L-15 medium was added to the cells, which were maintained for 24 h at 37°C. Luciferase activities were measured for the RSV-luciferase normalizing vector and radioactive CAT activity assays were performed by using cell extracts from transfected cells.

Results

Activity of the Chicken β-Globin Insulator in Transiently Transfected Nonerythroid and Erythroid Cell Lines. We tested the ability of sequences within the chicken β-globin insulator to affect enhancer activity in a transient expression assay. Several plasmids were constructed containing DNA segments from the chicken β-globin insulator (Fig. 2*A*). These included the original full-

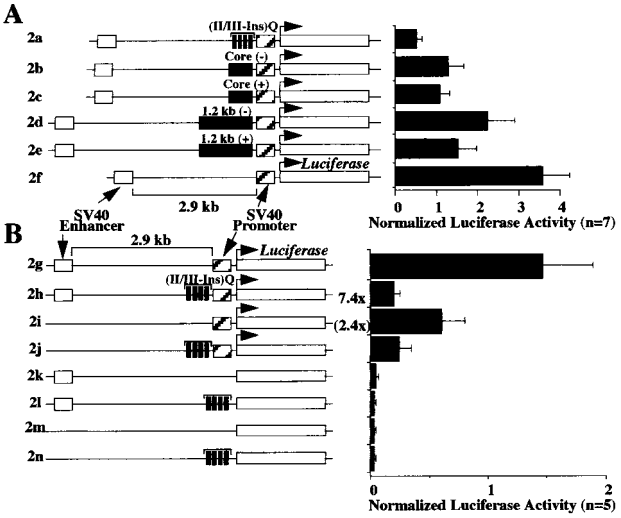

Fig. 2. (*A*) Transient activity in 6C2 cells of the different fragments of the chicken β-globin insulator. An enhancer-blocking assay was performed to test the ability of the 1.2-kb DNA fragment (constructs 2d and 2e), the core (250-bp; constructs 2b and 2c), and (II/III-Ins)Q to block the action of an enhancer (construct 2a). All constructs have a SV40 enhancer (open box) and a SV40 promoter (striped box) driving a luciferase reporter gene (construct 2f). Test fragments are located between the enhancer and promoter (constructs 2a–2e). The distance between the enhancer and its promoter is indicated. Error bars represent the standard error of the means (SEM) for seven experiments; (+) and (−) symbols show the 5′ to 3′ and 3′ to 5′ orientation of the fragments, respectively. (*B*) Transient activity of circular plasmids in 6C2 cells of the (II/III-Ins)Q element with various enhancer–promoter constructions in the pGL2 series of luciferase vectors. The reduction in reporter activity (7.4-fold) was caused by the insulator (construct 2h); the reduction in enhancer activity (2.4-fold) relative to the SV40 promoter alone (compare construct 2g with 2i). Data are the average of five independent experiments; error bars show the SEM here and in all figures.

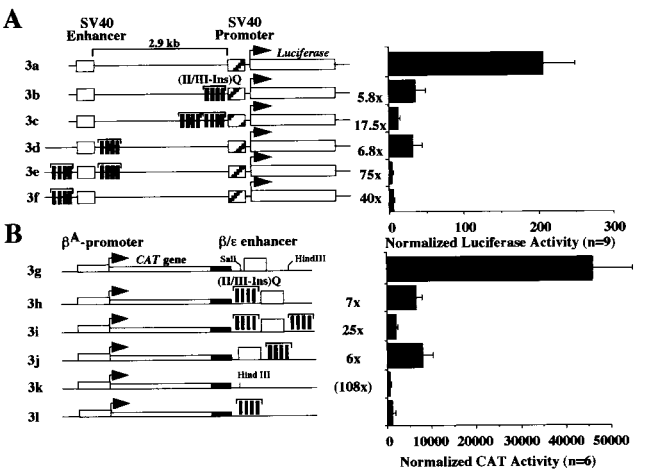

Fig. 3. Transient activity of (II/III-Ins)Q in 6C2 cells and 10-day-old embryonic erythrocytes. (*A*) Constructs in the pGL3 circular vector. Data are the average of nine independent experiments. (*B*) The chicken adult β-globin promoter and a minimal β/ε enhancer driving the expression of the *CAT* gene were used as reporter (construct 3g) (11). The number in brackets (construct 3k) represents the -fold of activation in the presence of the enhancer. The number to the side of each construct represents the -fold enhancer blocking. Data are the average of six independent experiments.

length 1.2 kb DNA fragment (constructs 2d and 2e), the 250-bp core insulator element (constructs 2b and 2c) previously shown to have enhancer-blocking activity (13), and the (II/III-Ins)Q element (construct 2a) containing four tandem copies of a 90-bp sequence, within the 250-bp core (Fig. 1) (15). To measure the effect of each element on the enhancer activity, these were introduced as closed circular plasmids into 6C2 cells (a transformed chicken erythrocyte precursor cell line) and QT6 cells (a transformed quail fibroblast cell line used as a source of nonerythroid cells). The DNA fragments were inserted between the SV40 enhancer and promoter elements of the pGL2-control vector (Fig. 2*A*, vector 2f). The 1.2-kb full-length insulator and the core insulator caused relatively small reductions of 2.4- and 3.3-fold in the reporter activity in the 5′ to 3′ orientation (i.e., in the same orientation as in the domain [Fig. 2*A*, vector 2e, (+)]. The (II/III-Ins)Q element was more effective, lowering luciferase activity 6.3-fold (Fig. 2*A*). This observation is consistent with the activity of these elements when stably integrated into the human erythroleukemia K562 cell line (13, 15). In fibroblasts, the 1.2-kb fragment in the 5′ to 3′ orientation induced a 1.75-fold reduction in the reporter activity (data not shown). The remaining constructs did not have significant activity, except that the (II/III-Ins)Q element again caused a 2.5-fold reduction in reporter activity, suggesting that this element is at least partially active in nonerythroid cells. We conclude that the 1.2-kb chicken β-globin insulator element and sequences within it are active in erythroid and nonerythroid cells and are capable of affecting transcriptional activity of reporters on a nonintegrated template.

Is the (II/III-Ins)Q an Enhancer or a Promoter Element in Transient Transfections? To examine whether the (II/III-Ins)Q contains an enhancer or promoter activity, we transfected 6C2 cells with reporter plasmids carrying (II/III-Ins)Q, in combination with the SV40 enhancer, promoter or both (Fig. 2*B*, constructs 2g, 2i, and 2k). We took advantage of the fact that SV40 enhancer–promoter elements are known to have only a weak stimulatory activity in erythroid cells. Consistent with earlier transient expression with the 250-bp core insulator (13), the (II/III-Ins)Q element did not have a promoter activity even in the presence of the SV40 enhancer (Fig. 2*B*, construct 2l). Furthermore, the already weak activity of the SV40 promoter was diminished about 3-fold by this element (Fig. 2*B,* construct 2j). Enhancer-blocking activity was also observed, but again the effects were small because the enhancer only induced a 2- to 3-fold stimulation of expression (Fig. 2*B*, construct 2i). The principal conclusion of these experiments is that the (II/III-Ins)Q element does not possess a promoter activity of its own in transient expression in these cells.

Transient Transfection in 6C2 Cells and 10-Day-Old Embryonic Erythrocytes. The constructions used above to look for possible stimulatory effects of the insulator were not suitable for studies of enhancer blocking because of their low levels of expression. We therefore took advantage of a different circular plasmid (pGL3-control, Fig. 3*A*, construct 3a) which possesses a stronger luciferase activity, and modified it for the purposes of these studies (*Materials and Methods*). The insulator-derived sequences were placed in different locations in relation to the SV40 enhancer–promoter (Fig. 3*A*). Three results of these experiments can be summarized: First, when a copy of (II/III-Ins)Q was placed "between" the enhancer and the promoter (i.e., between the 5′ end of the luciferase gene and the enhancer), the same level of blocking (5.8- and 6.8-fold) occurred regardless of whether the element was placed close to the enhancer or close to the promoter in these circular plasmids (Fig. 3*A*, constructs 3b and 3d). In addition, the insulator dosage correlated with the activity because two copies of the (II/III-Ins)Q element possessed even stronger blocking activity (17.5-fold; Fig. 3*A*, construct 3c). Second, this element had maximal blocking activity (75-fold) when the enhancer was flanked on both sides by the insulator (Fig. 3*A*, construct 3e). Third, when located "outside" (Fig. 3*A*, construct 3f), i.e., between the 3′ side of the luciferase

gene and the enhancer, there was still a significant reduction (40-fold) in reporter activity.

In stable transformation experiments, the insulator element can only block enhancer action when it lies between the enhancer and promoter. If the presence of an insulator on one side of the enhancer does not interfere with its ability to interact in the other direction within the circle, then in a circular plasmid, the enhancer will be completely blocked only when there is an insulator on both sides of it. Whether or not this occurs will depend on the detailed mechanism of enhancer and insulator action (Fig. 6). We find that the (II/III-Ins)Q element partially blocks enhancer activity when it is placed on one side or the other of the enhancer (Fig. 3*A*, constructs 3d and 3f, and Fig. 6), but maximum enhancer-blocking is observed only when the enhancer is flanked on both sides by the (II/III-Ins)Q element (Fig. 3*A*, construct 3e and Fig. 6). This result shows that within a circle, both sides of the enhancer must be blocked. Our data with the element only on one side of the enhancer imply that the enhancer "prefers" interaction with the promoter in one direction over the other (Fig. 3*A*, constructs 3d and 3f). It should be noted, however, that the distance between the enhancer and the promoter is shorter (by ≈1 kb) in the direction 3′ with respect to the luciferase poly(A) site (Fig. 3*A*, construct 3f). This difference in distance may explain the apparent preference of the enhancer.

To test the generality of the blocking activity, we performed similar experiments in an erythroid system: the chicken adult β^A-globin promoter and the minimal β/ε enhancer driving the *CAT* gene as a reporter in 10-day-old embryonic erythrocytes (Fig. 3*B*, construct 3g) (11, 18). In this context, the promoter activity was enhanced 108 times by the β/ε enhancer (compare Fig. 3*B*, constructs 3g and 3k). The (II/III-Ins)Q sequence blocked this strong enhancer activity (Fig. 3*B*, construct 3h). Again, when the (II/III-Ins)Q element was located on both sides of the enhancer, the blocking activity of this element was greatest (25-fold; Fig. 3*B*, construct 3i). We observed similar effects when the same plasmids were transfected into the chicken preerythroid cell line 6C2 although here all signals were lower (data not shown); thus, we confirmed the blocking activity of the (II/III-Ins)Q element in a nonintegrated context with different regulatory elements and in different cell types.

The 90-bp Insulator and the Effects of Mutating Two Distinct Protein Binding Sites. The (II/III-Ins)Q element consists of four copies of a 90-bp DNA sequence derived from a described 250-bp core DNA fragment of the 1.2-kb insulator element (Fig. 1). Like the larger fragments, repeats of the 90-bp sequence possess enhancer-blocking activity in the stable transformation assay, which measures the ability of the elements to reduce expression of a neomycin-resistance gene. We had used DNase I footprinting *in vitro* to identify within the 250-bp core fragment a set of nuclear-protein binding sites (13). The 90-bp subfragment of the core contains two of these binding sites, which we call FII and FIII. A combined deletion and functional analysis revealed that the major enhancer-blocking activity in stable transformation experiments comes from FII (15) and that this activity was attributable to the DNA-binding protein, CTCF. Taking advantage of these findings, we performed transient expression studies in 10-day-old embryonic erythrocytes with several versions of the 90-bp element (Fig. 4). We examined the effects of deletion of FII and FIII from the 90-bp fragment as well as a mutation of FII that abolishes binding of CTCF (Fig. 4, constructs 4d and 4f) (15). First, consistent with the results obtained with stably integrated constructs, in the transient transfections, four copies of the 90-bp insulator were stronger than one copy in blocking the enhancer (13-fold vs. 6-fold reduction; compare constructs 4b and 4c). Much of the observed enhancer-blocking activity was attributable to FII: a three-base mutation, which abolished DNA

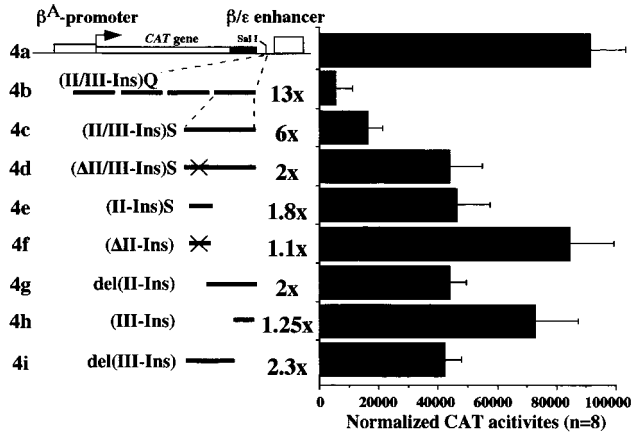

Fig. 4. Transient activity of a 90-bp minimal element in 10-day-old embryonic erythrocytes. The 90-bp fragment alone was tested in combination with different mutations of FII and FIII. Data are the average of eight independent experiments.

binding to FII, but retained FIII, resulted in loss of two thirds of the enhancer-blocking effect (Fig. 4, construct 4d). In transient assays, however, an FII subfragment alone had only a 1.8-fold effect (construct 4e) compared with a 6-fold reduction by a fragment containing both FII and FIII (construct 4c). This effect was completely abolished by the 3-bp mutation that eliminates CTCF binding (construct 4f). An FIII subfragment alone had no significant activity (construct 4h). Taken together, these results suggest either that the region between the two footprints contributes to the total activity of the 90-bp fragment or that some synergistic effect between FII and FIII exists. Although the 90-bp sequence also has three binding sites for the ubiquitous transcription factor Sp1 (13, 15), we found that mutating these sites had no effect on the enhancer-blocking activity of this sequence in transient assays (data not shown). These observations led us to conclude that most of the 90-bp enhancer-blocking activity derives from the interaction of CTCF with FII, although to reach the maximum enhancer-blocking activity in the 90-bp context, both FII and FIII are required.

Transient Transfection with Linearized Plasmids. As illustrated above, when situated in circular plasmids, different elements of the chicken β-globin insulator can only block enhancer-mediated activation completely when they surround the enhancer. One interpretation of this result is that in a circular construction, enhancer–promoter interactions blocked in one direction can still occur in the other. According to this view, enhancer-mediated activation is bidirectional: the enhancer can act independent of its position relative to the promoter, and furthermore, the restriction imposed by an insulator on one side of the enhancer does not influence enhancer action in the other direction. To test this idea, we repeated the experiments with linearized plasmids. Plasmids containing the chicken β^A-globin promoter and β/ε enhancer with the *CAT* gene as reporter were linearized with a restriction enzyme which leaves 1.9 kb of plasmid sequences on one side and 2.8 kb on the other (Fig. 5, construct 5a). On linear templates when the (II/III-Ins)Q element was located between the enhancer and the promoter, we observed a drastic reduction in the reporter activity (construct 5b); however, when this element was located outside of the enhancer (construct 5c), there was now no significant effect on reporter activity. Thus, unlike the results obtained with circular plasmids, on linear templates, the position of the insulator relative to the enhancer had a large impact on reporter activity. Southern blot analysis of the transfected linear DNAs suggested

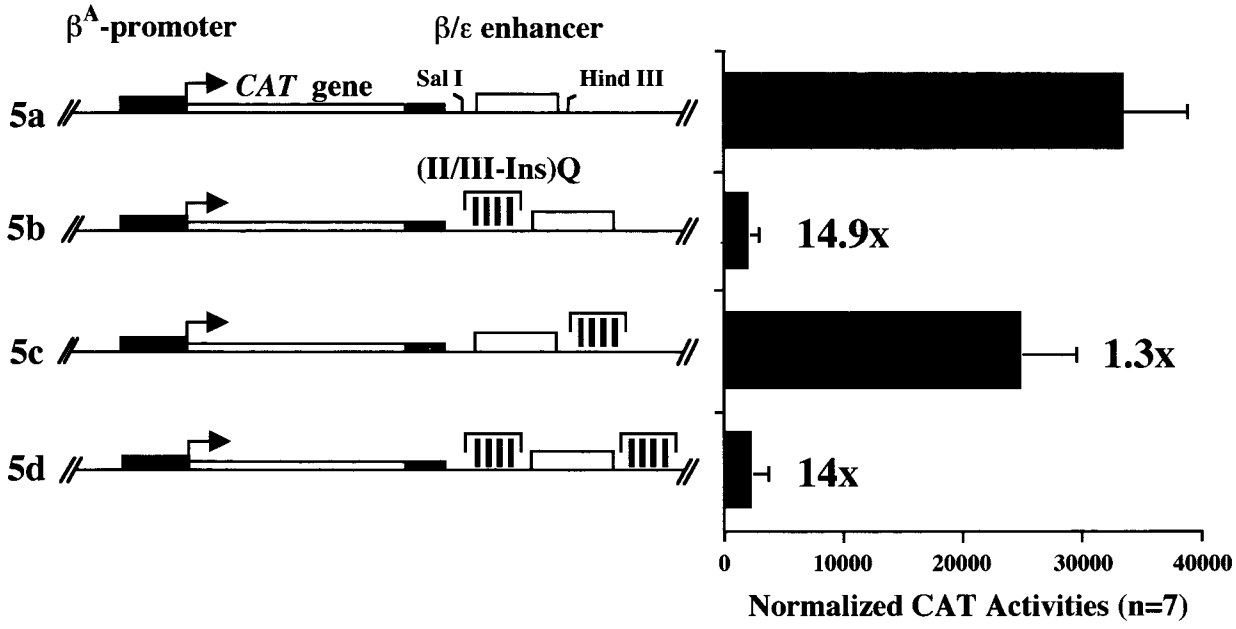

Fig. 5. Transient transfection of linearized plasmids into 10-day-old embryonic erythrocytes. The plasmid used for normalization is also linearized to monitor for variability that might arise from degradation. Data are the average of seven independent experiments.

that degradation was unlikely to account for these data, and consistent with this observation, in side-by-side comparisons, the activity levels observed with linear and circular reporters were similar (data not shown). Similar results were obtained in quail fibroblasts and a number of human cell lines (data not shown). We conclude that when the template is linearized, the enhancer-blocking element acts only when it lies between the enhancer and the promoter, exactly as was observed in stable transformation experiments and with other insulators (3, 15, 20).

Discussion

Boundary elements are DNA sequences that have been suggested to delimit independent chromatin domains (4, 21, 22). In the few examples that have been examined thus far, boundary elements act as insulators (2, 12, 14, 22). The properties of these elements are typically assayed by stable transformation into cells or organisms. Here we have made use of an assay based on transient transfection of vertebrate cells to examine the enhancer-blocking activity of the chicken β-globin insulator. Our results demonstrate that the 1.2-kb chicken β-globin insulator and the (II/III-Ins)Q element derived from it have position-dependent enhancer blocking activity in a nonintegrated reporter. This is most clearly demonstrated in experiments with linearized plasmids, in which we observe the same position-dependent enhancer-blocking activity found in an integrated context. In contrast, in circular plasmids, a complete block of enhancer-mediated activation requires that the enhancer be surrounded by insulator elements.

The concept of an insulator was operationally defined to encompass two activities (22). The first concerns the ability of an insulator to block the influence of an enhancer on a promoter. The second describes its ability to protect against position effects and act as a boundary between active and inactive chromatin. The *gypsy* insulator is a good example of the first type of insulator, although it is also capable of protecting the *mini-white* gene from position effects (4, 5). The *Fab-7* and *Mcp* boundary elements of the bithorax complex can also be included in this group (23). The second type of activity is exemplified by the *scs* and *scs′* elements, which flank the divergent *hsp70* genes at the 87A7 locus in *Drosophila* and appear to mark the boundaries of this domain (1). The 1.2-kb chicken β-globin insulator shares both characteristics: It protects against position effects in both transgenic flies and mice (12, 24) and chicken cell lines (14) and it also displays enhancer-blocking activity in both integrated and nonintegrated contexts (12, 13, 15, and this report).

Recently, we have shown that binding sites for CTCF, an 11 zinc-finger DNA-binding protein (25), play an essential role in the enhancer-blocking capacity of vertebrate insulators (15). In an integrated context, the binding of CTCF to the FII fragment of the chicken β-globin insulator is sufficient to initiate directional enhancer blocking. 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 (26), but when it interacts with sites in the *c-myc* oncogene, it causes repression (25). It is also capable of acting in synergy with certain thyroid hormone receptor binding sites both in repression and T3 induction (27). CTCF appears to be a multifunctional protein. By employing different subsets of its zinc fingers, it binds to quite varied DNA sequences (25) and in doing so may alter the nature of its interactions with cofactors, and thus, its ultimate biological effect. We show here that silencing and enhancer blocking are not easily distinguished on circular templates. It will be interesting to see how other CTCF sites behave when analyzed on linear templates.

The most striking observation arises from our comparison of the action of an insulator in circular versus linear templates. We believe that these results are in part a reflection of the way enhancers activate transcription. Two principal mechanisms have been suggested to explain the ability of enhancers to increase the rate of transcription from a promoter. The most familiar models, based largely on experiments in yeast, suggest that transcription factors bound to an enhancer interact directly with RNA polymerase, and/or its associated factors, and that this interaction increases the transcription rate perhaps by stabilizing the initiation complex at the promoter (28, 29). A commonly held view is that enhancers interact with promoters through a random collision event that results ultimately in the looping out of the intervening DNA. This is often called the looping model. In contrast to yeast, the distance between enhancer and promoter elements in higher eukaryotes can be

very large. Random collision at these distances becomes an unlikely event (22). This difficulty is circumvented in a second model which supposes that enhancer–promoter engagement occurs after tracking of one or both of these elements along the continuous DNA path between them (28). Courey *et al.* (31) have provided evidence for a bidirectional scanning model of enhancer action by showing that they could block activation within a plasmid by introducing psoralen cross-links into DNA between promoter and enhancer. For the greatest effect, it was necessary to introduce blocks on both sides of the enhancer. The placement of a particular plasmid sequence (presumably a cryptic insulator) between enhancer and promoter also gave rise to directional blocking. Similar results with circular plasmids have been obtained in other systems as described in the Introduction (6, 8–10).

These observations are in agreement with the enhancer-blocking results that we obtained in circular plasmids (Fig. 3*A*, construct 3e). In these experiments, the enhancer remains quite active when the insulator element is located only on one side (Figs. 3 and 6); the highest levels of blocking activity are found when the insulator is located on both sides of the enhancer. As observed previously with the *gypsy* insulator, the effect of the (II/III-Ins)Q insulator does not arise from silencing of the enhancer (20). This is demonstrated most clearly by our experiments with linearized plasmids. In those experiments, the (II/III-Ins)Q element blocks enhancer action only when it is located between the enhancer and the promoter (Fig. 5). Although these results seem to favor a tracking model, it must be kept in mind that transiently transfected plasmids may associate with structural elements in the nucleus (33) and therefore that steric interference with direct enhancer–promoter interactions may also play a role in enhancer-blocking activity.

Our observations also raise questions concerning the requirement of a chromatin context for the position-dependent enhancer-blocking activity of the chicken β-globin insulator. It has been demonstrated previously that transiently transfected plasmids acquire some degree of chromatinization and that nucleosomes can assemble on them (32–34). Indeed, the effects of histone acetyltransferase on transcription are apparent on transiently transfected plasmid templates (35). We do not know what relationship the partly chromatinized plasmid templates have to the transcriptionally active fraction, or indeed what fraction of all templates is active. It is therefore not possible to draw conclusions about the relevance of chromatin structure to the blocking activity in these experiments. The availability of a transient expression system that displays positional enhancer blocking activity may make possible an *in vitro* system in which the various proposed mechanisms can be tested more directly.

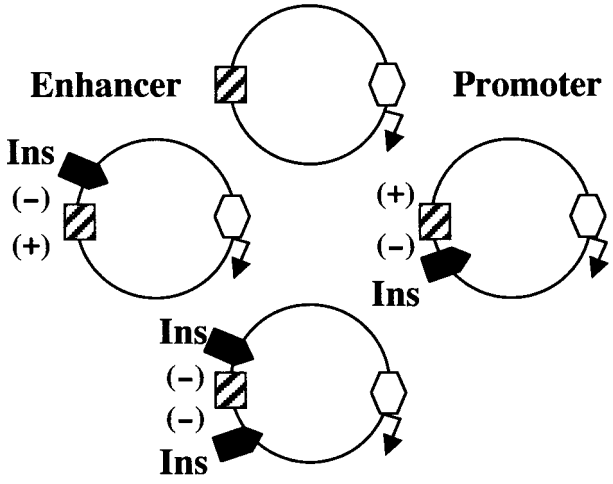

Fig. 6. Enhancer-blocking in circular plasmids. A scheme representing the enhancer-blocking activity of the (II/III-Ins)Q element as in the case of the experiments shown in Fig. 3. When (II/III-Ins)Q is placed between the enhancer and the promoter, the enhancer can in principle ''reach'' the promoter from the opposite side. Only when the (II/III-Ins)Q is located on both sides of the enhancer can the highest enhancer-blocking activity be reached.

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