

Mechanism of Chromosomal Boundary Action: Roadblock, Sink, or Loop?

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ABSTRACT

Boundary elements or insulators subdivide eukaryotic chromosomes into a series of structurally and functionally autonomous domains. They ensure that the action of enhancers and silencers is restricted to the domain in which these regulatory elements reside. Three models, the roadblock, sink/decoy, and topological loop, have been proposed to explain the insulating activity of boundary elements. Strong predictions about how boundaries will function in different experimental contexts can be drawn from these models. In the studies reported here, we have designed assays that test these predictions. The results of our assays are inconsistent with the expectations of the roadblock and sink models. Instead, they support the topological loop model.

EUKARYOTIC chromosomes are subdivided into a series of functionally, biochemically, and structurally autonomous domains. This was first recognized more than a half century ago in cytological studies on amphibian lampbrush chromosomes and insect polytene chromosomes (ALFERT 1954; GALL 1956). Over the intervening period, convincing evidence for the organization of chromosomes into discrete domains that have distinct and characteristic chromatin architectures has come from a combination of genetic, molecular, and biochemical experiments (for reviews, see UDVARDY 1999; GASZNER and FELSENFELD 2006; VALENZUELA and KAMAKAKA 2006). A classical example of an active domain is the 35-kb β -globin locus in chicken erythrocytes. The chromatin of this locus differs markedly from nearby transcriptionally repressed domains. In addition to an enhanced sensitivity to DNase I digestion compared to sequences in silent domains, β -globin chromatin has other distinctive properties including lower levels of the linker histone H5 and much higher levels of acetylated histones (BELLARD *et al.* 1980; STALDER *et al.* 1980; HEBBES *et al.* 1992; VERREAULT and THOMAS 1993). The patterns of methylation of histone H3 at lysine residues 4 and 9 in the β -globin domain also differ from that of silenced domains

(LITT *et al.* 2001). The features that distinguish the active globin domain from the surrounding inactive domains are evident not only elsewhere in the chromosomes of vertebrates, but also in the chromosomes of many other eukaryotes.

The organization of eukaryotic chromosomes into domains having distinct chromatin architectures and genetic activities requires a mechanism for delimiting the domains. Special elements called boundaries or insulators serve this purpose. These elements define the limits of chromosomal domains and function to establish independent units of gene activity. Elements having these properties were first discovered in *Drosophila* but have since been found in many organisms ranging from yeast to humans (GASZNER and FELSENFELD 2006; VALENZUELA and KAMAKAKA 2006). While mutations in boundary elements can be associated with unusual phenotypic effects (KEPPY and WELSHONS 1977; GYURKOVICS *et al.* 1990), the biological activities of most boundaries have been investigated using two different transgenic assays. The first assay postulates that boundaries function to establish autonomous units of genetic activity and it tests whether these elements can insulate reporter genes against positive and negative chromosomal position effects. It was found that the reporter must be bracketed by boundary elements to guard against position effects, while a single element either upstream or downstream of the reporter was unable to insulate (KELLUM and SCHEDL 1991; CHUNG *et al.* 1993). The second assay was based on the fact that enhancers and silencers are not only rather promiscuous in their regulatory interactions but are also able to regulate genes located many kilobases away, independent of orientation and position. This meant that there must be mechanisms in place that constrain the regu-

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latory activities of enhancers/silencers to their designated target genes. It was suggested that this regulatory problem might be solved if boundaries functioned to restrict enhancer activity to the chromosomal domains in which they reside. This idea was tested using a blocking assay in which boundaries were placed between an enhancer or silencer and a target promoter (HOLDRIDGE and DORSETT 1991; GEYER and CORCES 1992; KELLUM and SCHEDL 1992; CHUNG *et al.* 1993). It was found that boundaries can block regulation when interposed between an enhancer or silencer and the promoter, while they have no effect when located distal to the enhancer or silencer.

Like enhancers/silencers, boundaries are now known to be ubiquitous components of the genome in multicellular eukaryotes. For example, recent studies on the chromosomal distribution of the boundary protein CTCF in humans have identified >13,000 *in vivo* binding sites (KIM *et al.* 2007; XIE *et al.* 2007). In addition, >14,000 putative insulators have been reported in the *Drosophila* genome (BUSHEY *et al.* 2009; NEGRE *et al.* 2010). As expected for elements that function to prevent adventitious regulatory interactions, the CTCF sites in the human genome are enriched in intergenic regions that separate differentially expressed genes or gene clusters. CTCF-dependent boundaries in *Drosophila* have also been shown to demarcate the *cis*-regulatory domains of the homeotic Bithorax and Antennapedia complexes (MOON *et al.* 2005; HOLOHAN *et al.* 2007; NEGRE *et al.* 2010).

While the importance of boundary elements in delimiting units of independent gene activity has now been well established, how they function to block enhancers (and silencers like the fly Polycomb Response Elements) is not well understood. Three different models have been proposed to explain how boundaries restrict the activity of enhancers. For two of these, it is assumed that the mechanism used by enhancers (or silencers) to act over large distances will dictate how boundaries function and both of these models are based on currently popular ideas for how enhancers contact their target genes (WEST *et al.* 2002; KUHN and GEYER 2003; SCHEDL and BROACH 2003; GASZNER and FELSENFELD 2006). In the first, enhancers (silencers) provide entry points for regulatory machines that track along the chromosome toward the promoter (see Figure 1A). This model has recently come back into favor with the realization that both gene activation and silencing depend upon histone modifications such as methylation and acetylation (BERGER 2007). Moreover, as would be expected for complexes that processively alter chromatin structure, many of these histone modifications are not limited to the target promoter, but instead appear to encompass the entire regulatory domain (NOMA *et al.* 2001; SCHWARTZ *et al.* 2006; TOLHUIS *et al.* 2006). In this model, boundary elements function as roadblocks, blocking the movement of the regulatory complexes

along the chromatin fiber (Figure 1A). Roadblock activity could be entirely passive, generated by the assembly of a large nucleoprotein complex at the boundary that disrupts the regular chromatin architecture and prevents the passage of the regulatory machinery (BI and BROACH 1999). The roadblock could also recruit enzymatic activities that locally modify chromatin around the boundary and thus help promote the maintenance of an inactive or active state (depending upon whether the roadblock is blocking an enhancer or silencer, respectively). Evidence for such chromatin-modifying activities has been reported for several yeast and vertebrate boundaries that function to block the spread of repressive heterochromatin (OKI and KAMAKAKA 2002; WEST *et al.* 2004). In this regard, it is interesting to note that in the case of the boundary at the 5' end of the β -globin locus blocking the spread of heterochromatin is separable from blocking enhancer action. The former appears to involve a "barrier" activity that maintains a local active chromatin environment in the vicinity of the boundary element, while the latter depends upon the highly conserved CTCF boundary factor. A similar distinction has been drawn for the *gypsy* transposon *su(Hw)* boundary (KURSHAKOVA *et al.* 2007).

The second model for enhancer action postulates that enhancers and silencers contact their target genes by a looping mechanism (see Figure 1B). In this model, the looping of the chromatin fiber brings DNA binding proteins and other factors associated with the enhancer into close proximity with the target promoter (BLACKWOOD and KADONAGA 1998). These factors can then recruit or contact components of the transcriptional apparatus such as Mediator complex, transcription factor II D (TFIID), or PolII or modify the chromatin organization of the promoter and flanking regions to activate or repress transcription. There is now considerable evidence from chromatin conformation capture (3C) experiments that enhancers and their target promoters are in close contact with one other *in vivo* (TOLHUIS *et al.* 2002; ENGEL *et al.* 2008). In this model, the interposed boundary elements must be able to prevent the looping enhancer from making contact with the target gene (Figure 1B). Unlike the roadblock model, preventing the looping enhancer from establishing productive contacts with the target gene demands an active mechanism (KUHN and GEYER 2003). It has been suggested that the boundary captures and retains the looping enhancer before it can make contact with the target gene by acting as a promoter decoy or sink (GEYER 1997; BLACKWOOD and KADONAGA 1998). The capturing process would likely require the establishment of direct protein:protein contacts between factors associated with the enhancer and the boundary. As boundaries show little specificity in the sorts of enhancers (or silencers) that they can block, this model would require that boundary proteins are quite promiscuous in their potential protein:protein interac-

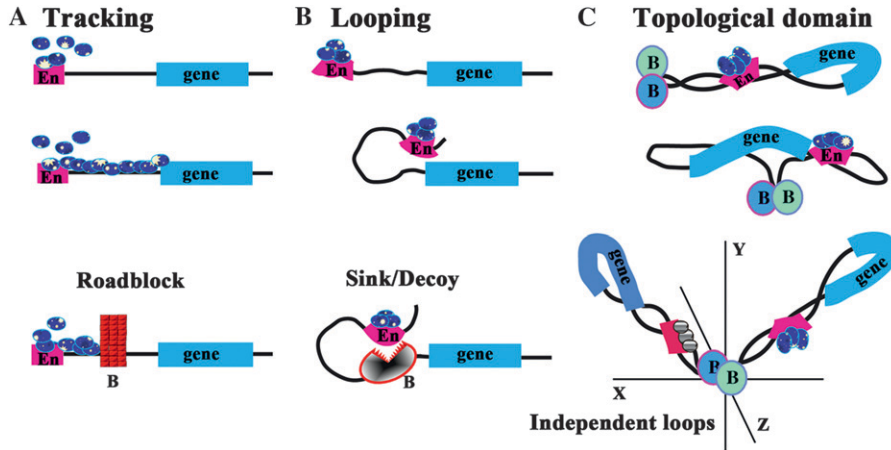


FIGURE 1.—Enhancer blocking in the roadblock, sink, and loop domain models. (Top to bottom) (A) In the tracking model, regulatory factors (blue ovals) first assemble at the enhancer (or silencer) (En) and then move processively along the chromatin fiber toward the promoter of the regulatory target. In this model, boundaries (B in section A) act as roadblocks or barriers, preventing the processive movement of the regulatory factors. (B) In the looping model, regulatory factors associate with the enhancer (or silencer) (En). These bound factors then contact the promoter of the regulatory target by the looping over of the chromatin fiber. In this model, boundaries (B in section B) act as sinks,

capturing (or repelling) the looping enhancer/DNA complex before it can make contact with the target promoter. Unlike the roadblock model where boundary action is passive, boundaries (B) must actively block regulatory interactions in the looping model. (C) In the topological loop domain model, the chromosome is subdivided by boundaries into a series of topologically independent loops. Enhancers/silencers (En) would contact their regulatory targets by the sliding of the chromatin fiber in the loop against itself (which formally is a combination of both tracking and looping). In this illustration, the enhancer is located 3' to the transcription unit rather than 5' as in A and B. Genes are topologically isolated from regulatory elements in adjacent loops, and contacts can only be made by a search in three-dimensional space. In this and subsequent figures, the proximal and distal endpoints of each looped domain are delimited through interactions between boundaries. The endpoints of the looped domain could also be defined by boundary interactions with the nuclear matrix, the chromosome scaffold, or insulator bodies.

tions. In other versions of this model, boundaries repel instead of capturing the looping enhancer.

The third mechanism for boundary function is based on the notion that boundaries subdivide the chromosome into a series of topologically independent domains either by interacting with each other or with some nuclear structure(s) (UDVARDY and SCHEDL 1984; BLANTON *et al.* 2003; BONDARENKO *et al.* 2003; SCHEDL and BROACH 2003; SPLINTER *et al.* 2006). This idea was first suggested by studies from the Worcel and Laemmli laboratories in the 1970s, which showed that eukaryotic chromosomes are organized into DNA loops of 10–100 kb in length (PAULSON and LAEMMLI 1977; WORCEL and BENYAJATI 1977). In this model, restraining the action of enhancers/silencers and protecting against both positive and negative chromosomal position effects would be a consequence of the subdivision of the chromatin fiber into topologically independent loops. Enhancers or silencers located within a single looped domain could be brought into contact with their target genes by the sliding of the chromatin fiber in the loop against itself (see Figure 1C) or by intraloop contacts. While interactions within a loop are physically constrained, transient contacts between regulatory elements in one loop and a target gene in another loop would require searching in three-dimensional space. In addition, the possibility of independent movement in three-dimensional space could make it more difficult to establish or sustain productive interactions between regulatory elements in one looped domain and potential target genes in another (RIPPE 2001; WEST and FRASER 2005). The idea that boundaries physically

restrict enhancer interactions with regulatory targets is supported by chromosome conformation capture (3C) experiments in the mouse *H19/Igf2* locus (ENGEL *et al.* 2008; NATIVIO *et al.* 2009).

The roadblock, sink/decoy, and looped domain models make straightforward predictions about the behavior or functioning boundary elements in different experimental contexts. In the studies reported here, we have used the postulated properties of boundary elements in each model to design assays that test these predictions and point to the likely mechanism of action.

MATERIALS AND METHODS

Fly methods: Flies were grown on standard cornmeal agar. All crosses were done at 22°. *su(Hw)^o/TM6*, *Ubx*, *su(Hw)^f* mutants were generously provided by Victor Corces. Transgenic lines were analyzed in a background maternally and zygotically mutant for *su(Hw)* (Figure 6).

Cloning of Pelement constructs: The following boundary fragments were used in the enhancer-blocking assays described in this article:

Fab-7, in the domain definition (*ftz-LacZ*) assay, a 1.7-kb *ApaI*–*NcoI* *Fab-7* fragment was used, to allow comparison with the results reported (HAGSTROM *et al.* 1996). In the triple boundary (mini-*white*) assay, an ~1.5-kb *Fab-7* fragment was used.

Fab-8, an 800-bp *Fab-8* fragment was amplified with the following primers:

F8 4620 RI, GGCACAATCAAGAATTCGTTGG

F8 3445 Bam: TGCCTCCGGATCCCCGACGGCTGAC.

scs, a 1.7-kb *scs* fragment from (VAZQUEZ and SCHEDL 1994) was used.

scs', a 450-bp *BamHI*–*EcoRI* fragment from (KELLUM and SCHEDL 1992) was used.

su(Hw), a 370-bp fragment derived from an *XmnI*-*Bsp1286* fragment of the *gypsy* retrotransposon was used (HAGSTROM *et al.* 1996). This fragment contains 12 degenerate binding sites for Su(Hw).

The boundary reporter transgenes used in these studies were:

Triple boundary (mini-white) constructs: XN-XN is described in detail in HAGSTROM *et al.* (1996). The XN vector has unique cloning sites upstream (*KpnI*) and downstream (*XhoI*, *NotI*) of a minimal *white* enhancer that drives *white* expression in the eyes and in the testes. For a detailed map of XN, see supporting information, Figure S1).

X8-X8 is a derivative of XN, in which the downstream *scs'* boundary has been replaced with an 800-bp *Fab-8* fragment (see above). To construct X8, XN was digested with *EcoRI*, the *P*-element backbone and *white* enhancer/mini-*white* fragments were separately gel purified. The *P*-element backbone was recircularized and then digested with *EcoRI*, *BamHI* to remove *scs'*. An *EcoRI*, *BamHI* *Fab-8* fragment was ligated into the *P*-element backbone. This plasmid was then cut with *EcoRI*, and the *white* enhancer/mini-*white* *EcoRI* fragment was added back into the plasmid in the proper orientation. Other boundary elements were then cloned into the unique restriction sites upstream (*KpnI*) and downstream (*XhoI*, *NotI*) of the *white* enhancer.

Domain definition (*ftz-LacZ*) constructs: pCfhL-pCfhL is described in detail in HAGSTROM *et al.* (1996). pCfhL contains two *fushi-tarazu* (*ftz*) enhancers, the upstream element (UPS), and the neurogenic enhancer (NE). Like XN, pCfhL also has unique cloning sites upstream (*KpnI*) of the *ftz* enhancers (between the enhancers and mini-*white*), and downstream of the *ftz* enhancers (between the enhancers and *LacZ*). For a detailed map of pCfhL, see Figure S1).

pCfhL(*SceI*)-pCfhL(*SceI*) is identical to pCfhL, except a pair of *I-SceI* sites have been introduced on either side of *scs'*. To construct pCfhL(*SceI*), an *I-SceI* flanked linker with unique *SpeI* and *NsiI* sites was generated. The *I-SceI* linker also had *XhoI* and *MfeI* sites 5' to the *I-SceI* sites, and *SfiI* and *XbaI* sites 3' to the *I-SceI* sites. The sequence of the linker was (*I-SceI* sites in boldface type):

GTTACTCGAGCAATTGATTACCCTGTTATCCCTACTAGTGATGATGATGATGATGCAATTACCCTGTTATCCCTAGGCCATATGGCCGCAGCGCCATATAGGCCTCTAGAATTG.

The *I-SceI* linker was cloned into the *XhoI*, *XbaI* sites of pBluescript to generate pBS-*SceI*. Next, pCfhL was cut with *XhoI*, and then partially digested with *EcoRI*, to isolate an *hsp70* promoter-*LacZ-hsp70* trailer fragment. This fragment was cloned into the *XhoI*, *MfeI* sites of pBS-*SceI*. The *XhoI*, *SfiI* *hsp70* promoter-*LacZ-hsp70* trailer-*I-SceI* linker fragment was then liberated and cloned into an *XhoI*, *SfiI* cut pCfhL backbone to generate pCfhL-*SceI*-linker. *scs'* was amplified out of pCfhL with the following primers and then cloned into pCRII-TOPO (Invitrogen) in order to flank *scs'* with *SpeI* sites:

scs' for, CGGGAATTCCAACAAAACTTTGC;

scs' rev *SpeI*, ACTAGTCTGTGAAAATAAAATGCCGT.

The *scs'* *SpeI* fragment was then cloned into *SpeI* cut pCfhL-*SceI*-linker plasmid in the same orientation as *scs'* in pCfhL to generate pCfhL(*SceI*).

Other boundary elements were then cloned into the unique restriction sites upstream (*KpnI*) and downstream (*XhoI*, *NotI*) of the *ftz* enhancers.

P-element-mediated transformation: A total of 0.5 mg/ml of DNA was co-injected with a P-turbo helper plasmid into *w¹* embryos, and transformants were selected by the presence of eye color. Individual transformants were then backcrossed to

w¹ flies for at least two generations and then balanced over *Bins*, *CyO*, or *TM3Ser*, to create a balanced stock and to determine the chromosome of insertion.

Deleting *scs'* using the *I-SceI* nuclease: To delete *scs'* from pCfhL(*SceI*)(*X-Fab7-scs'*) and pCfhL(*SceI*)(*Fab-8-Fab7-scs'*), flies carrying these transgenes were crossed to *w; TM3, Sb, P{4}72C* flies, which express the *I-SceI* nuclease under the control of the *Drosophila Ubiquitin (Ubi-p63E)* promoter (PRESTON *et al.* 2006). Potential deletion events were rebalanced and screened by PCR for deletion of *scs'*. The following primers were used to confirm the deletion of *scs'*:

pCfhL*SceI*-F2, CGATGGATATTCAGGTGCCAA;

pCfhL*SceI*-R1, GAGTGAGACAGCGATATGATTGT.

Scoring eye color phenotypes in the *white* enhancer-blocking assay: Balanced transgenic male flies were crossed to *w¹* females, and progeny hemizygous for the transgene were scored for levels of mini-*white* expression. Since age and sex both affect levels of mini-*white* expression, comparisons were made only between flies of the same sex and age. For X-linked transgenes, only females were scored, as the dosage compensation machinery seems to override enhancer blocking in males (VAZQUEZ and SCHEDL 1994). To determine whether the transgenic lines had a blocking or nonblocking phenotype and to allow comparison with the data from VAZQUEZ and SCHEDL (1994), HAGSTROM *et al.* (1996), and SCHWEINSBERG and SCHEDL (2004), four reference lines were used. Nos. 89.98.1 and 101.93.1 are two lines that have the lightest eye color previously classified as nonblocking (SCHWEINSBERG *et al.* 2004), while nos. 110.115A and 110.185A have the darkest eye color previously classified as blocking. Eye photos were taken with a Nikon FX-35WA camera and Nikon HFX-IIA photosystem on a Nikon SMZ-2T dissecting microscope.

β -Galactosidase stainings: To determine levels of β -galactosidase expression, transgenic males were crossed to *w¹* females. Embryos 0–15 hr old were collected from yeasted apple juice plates and stained as previously described (BELLEN *et al.* 1989; HAGSTROM *et al.* 1996), with the following modifications. Heptane was saturated with glutaraldehyde by mixing 10 ml heptane, 7.5 ml PBS, and 2.5 ml glutaraldehyde and vortexing vigorously. The phases were then allowed to separate and the top layer, containing glutaraldehyde-saturated heptane was added to a multi-well dish containing the embryos. Embryos were fixed for 20 min at room temperature, shaking. Embryos were stained for 20–24 hr, then transferred to 30% glycerol and left overnight. Embryos were then mounted on glass slides and pictures were taken using a Nikon DXM200F digital camera on a Nikon Microphot-SA light microscope. Comparisons were made between embryos stained in parallel, in the same dish. Positive and negative control lines were also used in each staining. Lines containing either no insert, or a random DNA insert in the blocking position, 56.33.1 and 25.129.1, respectively, were used as positive controls for β -galactosidase staining, and as standards for a nonblocking phenotype (HAGSTROM *et al.* 1996). Lines containing a 1.2-kb *Fab-7* insert, 117.17B, 117.31B, and 117.3B, were used as negative controls for β -galactosidase staining, and as standards for an enhancer-blocking phenotype (SCHWEINSBERG and SCHEDL 2004).

In situ hybridizations: *In situ* hybridizations were done as previously described (TAUTZ and PFEIFLE 1989). Briefly, a probe for *white* was prepared by *in vitro* transcription using T7 polymerase in the presence of digoxigenin (DIG)-labeled dNTPs (Roche). The *white*-containing plasmid was obtained from Jumin Zhou. Embryos 0–15 hr old were collected on apple juice plates. Embryos were washed onto 40- μ m mesh cell strainers (BD Falcon) with distilled water (dH₂O) and then

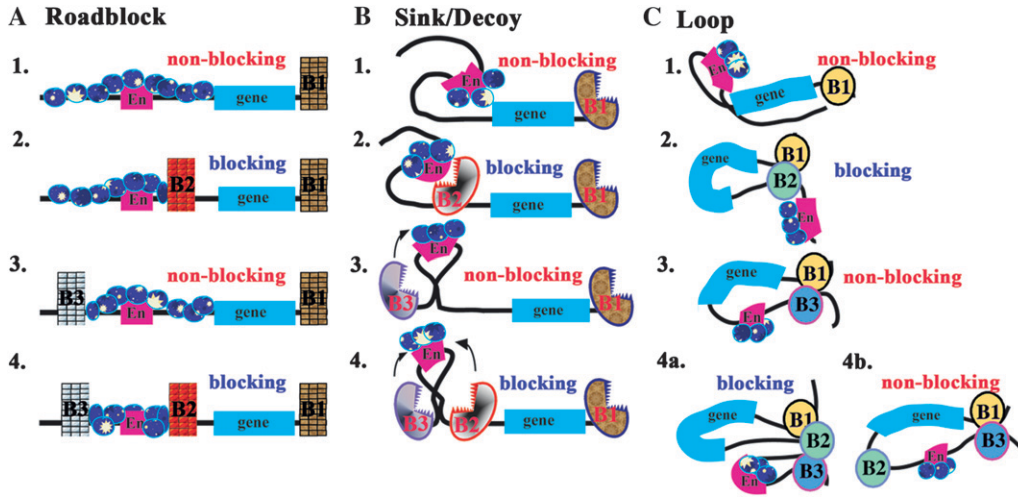


FIGURE 2.—Triple boundary assay. The starting transgene used for the triple boundary assay has a *white* reporter (“gene” indicated by blue line) and the *white* enhancer (En, red line). As shown in A–C1, a boundary (B1), either *scs'* or *Fab-8*, is placed downstream of the reporter. Additional boundaries are then introduced into the transgene in between the enhancer and the reporter and upstream of the enhancer as indicated in 2, 3, and 4. As shown in A–C1, the *white* enhancer is expected to activate *white* (non-blocking) in all three models

when the transgene contains only a single boundary downstream of the reporter. Likewise in A–C2, a boundary placed between the *white* enhancer and reporter (B2) will block activation (blocking) in all three models. As shown in 3, no blocking (nonblocking) will be observed when a boundary is placed upstream (B3) of the *white* enhancer in the roadblock (A3) and loop (C3) models. In the sink (B3) model, the upstream boundary should have the potential to capture (arrow) the looping enhancer before it can contact the promoter and thus some attenuation of enhancer activity could be observed. In 4, boundaries are placed upstream of the enhancer (B3) and in between the enhancer and the reporter (B2). In the roadblock model (A4), the addition of an upstream boundary (B3) should have no effect on the blocking activity (blocking) of the interposed (B2) boundary. In the sink/decoy model (B4), flanking the enhancer with boundaries is predicted to have no effect or increase blocking activity (see arrows in 4). In the loop model, blocking activity will be maintained if the enhancer and gene are in separate loops (via boundary interactions as illustrated, or by interactions with the nuclear matrix/chromosome scaffold). In C4b, blocking will be lost (nonblocking) if the loop is defined by the upstream (B3) and downstream boundaries (B1) (Note that the 3' end of the *white* reporter also contains the recently described *wari* insulator (CHETVERINA *et al.* 2008), though it is not indicated here. The presence of *wari* does not alter the predictions of each model. Moreover, this boundary does not appear to insulate in combination with *scs* or *Fab-7*).

dechorionated by submerging in bleach (Na hypochlorite 7%) for two minutes. Embryos were washed several times with dH₂O and then fixed in 4% paraformaldehyde in 1:1 PBS:heptane in a glass scintillation vial for 20 min at room temperature, shaking. After shaking, the bottom (aqueous) layer was removed, and 5 ml of 100% methanol was added. The vials were then shaken and vortexed vigorously, and embryos that settled on the bottom of the vial were transferred to a microfuge tube. Embryos were then rehydrated in a stepwise manner in 25, 50, 75, and 100% PBST in methanol. Following rehydration, embryos were washed five times with PBST and then treated with 10 µg/ml proteinase K (in PBST) for 2 min. Embryos were then refixed in 4% formaldehyde in PBS for 20 min at room temperature, while rocking. The embryos were then washed thoroughly with PBST, washed once with 1:1 PBST:hybridization buffer (50% formamide, 5× SSC, 50 µg/ml heparin, 0.1% Tween 20, 100 µg/ml sonicated salmon sperm DNA), and allowed to prehybridize in hybridization buffer for 2 hr at 55°. The DIG-labeled *white* probe was diluted 1:100, heated to 80°, added to the embryos, and incubated at 55° overnight to hybridize. The probe was then removed and the sample was washed extensively with hybridization buffer, followed by 1:1 PBST:hybridization buffer, and then five times with PBST. The embryos were then probed with 1:2000 HRP-conjugated anti-DIG antibody (Roche) for 1.5 hr. Upon removal of the antibody, the embryos were washed extensively with PBST and then washed twice with developing solution (0.1 M NaCl, 0.1 M Tris-HCl pH 9.0, 0.05 M MgCl₂, 0.1% Tween 20). The embryos in developing solution were transferred to a glass dish and 20 µl of NBT/BCIP solution was added. *In situ* were developed for between 30 and 120 min. Comparisons were made between groups of embryos that were developed

together for the same amount of time. The reaction was stopped by washing twice with PBST. The stained embryos were dehydrated in a series of 25, 50, 75, 90, 95, and 100% ethanol (in PBS) washes, and then washed twice with 100% ethanol. All traces of the ethanol were removed, and the embryos were incubated in 1 ml of methyl salicylate overnight. Embryos were then mounted on glass slides in 100 µl of Permount (Fischer) and pictures were taken using a Nikon DXM200F digital camera on a Nikon Microphot-SA light microscope.

RESULTS

Triple boundary: *Experimental design:* The enhancer-blocking assay tests the ability of a boundary element to prevent regulatory interactions. As illustrated in Figure 2, the starting reporter gene is typically flanked by a downstream boundary (B1) to protect against potential position effects at the site of insertion (1). In all three models, regulatory interactions are blocked when a boundary (B2) is interposed between the enhancer (2 in Figure 2, A–C) and the reporter. Both the roadblock and loop models predict that placing a boundary (B3) upstream of the enhancer instead of in between will have no influence on regulatory interactions (3 in Figure 2, A and C). In the sink/decoy model, boundaries capture or otherwise actively interfere with the regulatory element and, thus, an upstream boundary might be expected to attenuate, at least modestly, enhancer action

on the reporter (3 in Figure 2B). Since this has not been observed, it is assumed either that enhancer activity is in excess or that boundaries might repel rather than capture the looping enhancer.

It is possible to distinguish between the three models by determining what happens when a boundary located in between the enhancer and promoter is “challenged” by placing a boundary upstream of the enhancer. In the roadblock model, the ability to stop the processive “tracking” of regulatory factors from the enhancer toward the promoter is an intrinsic feature of each boundary and does not depend upon the activity of other nearby boundaries. Thus, the addition of an upstream boundary (B3) should have no effect on the blocking activity of the interposed boundary (B2), and full blocking activity should be retained (4 in Figure 2A). In the sink model, blocking activity is also an intrinsic property of the boundary and depends only upon the ability of the interposed boundary to capture or repel the looping enhancer before it can contact the promoter (4 in Figure 2B). However, since the enhancer would be bracketed by elements that act as sinks or repellents, the addition of an upstream boundary (B3) could accentuate the blocking activity of the interposed boundary (B2).

The loop model differs from the other models in that blocking activity does not depend solely on the intrinsic properties of the boundary element. Instead, because two boundaries are required to define the endpoints of a looped domain, blocking activity will be determined by how a boundary functions in relation to or in combination with the other boundaries in the transgene and with boundaries in the surrounding chromosomal environment. Since the boundaries (and their DNA sequence contexts) are different, some boundary combinations will be better matches than others and for this reason the outcome of the triple boundary assay will depend upon boundary competition. Additionally as the competition between the boundaries included in the transgene will be influenced by endogenous boundaries near the site of insertion, position effects are also to be expected. If the favored boundary combination generates one loop containing the enhancer and a second loop containing the reporter, blocking activity in the triplet (4a in Figure 2C) will be retained. In contrast, if the favored boundary combination generates a loop containing both the enhancer and the reporter (4b in Figure 2C), blocking activity will be lost. The factors that determine which boundary combinations are used to define the regulatory domains will ultimately depend upon how loops are generated. As suggested by MIRKOVITCH *et al.* (1984), YUSUFZAI *et al.* (2004), and others (RAZIN *et al.* 1981; COCKERILL and GARRARD 1986; LOC and STRATLING 1988), two boundaries could generate a loop by interacting with sites in the nuclear matrix or chromosomal scaffold. Alternatively, as proposed by UDVARDY *et al.* (1985), loop formation could be dictated by direct

physical interactions between boundary elements. Yet another model for loop formation would be the recruitment of boundaries into specialized, boundary-specific structures, such as the “insulator bodies” (GERASIMOVA *et al.* 2000, 2007). For simplicity, loop formation is illustrated in this and subsequent figures (Figures 2, 5, 7, and 8) by physical interactions between the boundaries that define the endpoints of the looped domain.

To test these predictions, we used a *white* reporter and enhancer to monitor regulatory interactions (VAZQUEZ and SCHEDL 1994). For boundaries, we selected *scs* and *scs'* from the 87A7 heat-shock locus (UDVARDY and SCHEDL 1984; KELLUM and SCHEDL 1991), *Fab-7* (HAGSTROM *et al.* 1996) and *Fab-8* (BARGES *et al.* 2000) from the Bithorax complex (BX-C), and *su(Hw)* from the *gypsy* transposon (GEYER and CORCES 1992). These boundaries differ in the DNA binding proteins thought to be important for their activity: *Zw5* contributes to *scs* activity (GASZNER *et al.* 1999), while *scs'* requires boundary element-associated factor 32 (BEAF) (ZHAO *et al.* 1995). *Fab-7* is unusual in that different factors confer boundary activity at different stages of development (SCHWEINSBERG and SCHEDL 2004). In the early embryo, the Elba factor seems to be especially important, while boundary activity later in embryogenesis and in the adult depends upon distinct, but as yet unknown factors (AOKI *et al.* 2008). The other Bithorax boundary *Fab-8* is a CTCF-dependent boundary (MOHAN *et al.* 2007). Finally, the *su(Hw)* element requires the DNA binding protein *su(Hw)* as well as a collection of other proteins [Mod(mdg4), CP190, and dTopors] (PAI *et al.* 2004; CAPELSON and CORCES 2006; GASZNER and FELSENFELD 2006).

An interposed scs retains blocking activity when challenged with upstream boundary: In the first experiment, *scs'* was placed downstream (B1) of *white*, while *scs* was interposed (B2) between the 1-kb *white* enhancer and reporter (Figure 3A; also see detailed map in Figure S1). In this configuration, *scs* effectively blocks the *white* enhancer, and in all transgenic lines examined the flies had a yellow/orange eye color phenotype (VAZQUEZ and SCHEDL 1994). As the upstream (B3) boundary, we used either *su(Hw)* or *Fab-8*. In the roadblock and sink models, placing either of these boundaries upstream of the *white* enhancer should have no effect on blocking activity or might even strengthen it. As for the loop model, 3C experiments indicate that the endogenous *scs* and *scs'* are physically associated *in vivo* (BLANTON *et al.* 2003). If these *scs*–*scs'* interactions are recapitulated in the triple boundary transgene or if the two elements generate loops by interacting with sites in the nuclear matrix or by targeting to insulator bodies, the blocking activity of *scs* should be unaffected by the upstream *su(Hw)* or *Fab-8* boundaries (Figure 2, C4a). However, it is also possible that *scs'* will preferentially combine with *su(Hw)* or *Fab-8*, looping out *scs*. In this case, the enhancer and reporter would be in the same loop (Figure 2, C4b) and blocking activity would be lost.

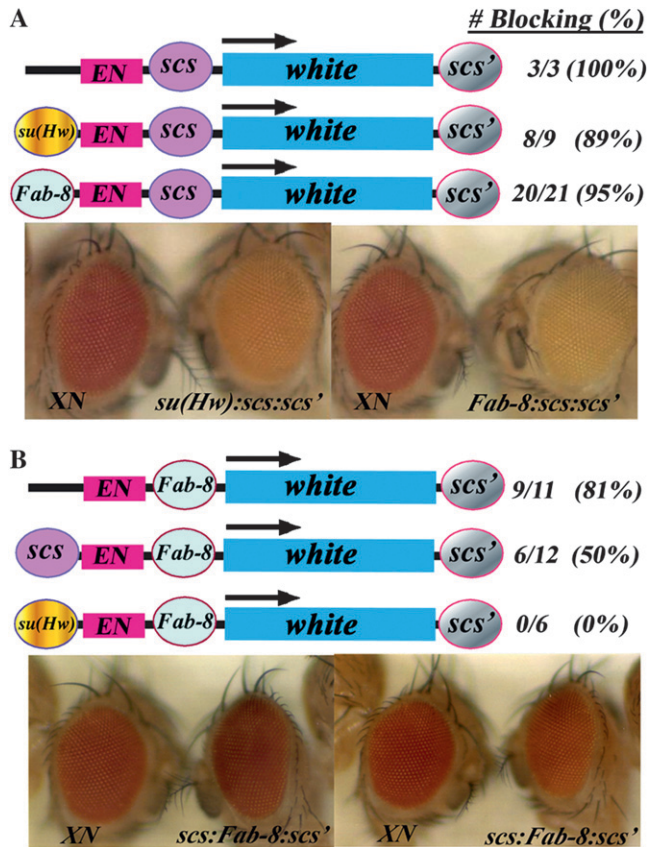


FIGURE 3.—Triple boundary with *scs* or *Fab-8* in the middle. (A) *scs* in between the *white* enhancer and the *white* reporter. When *scs* is interposed between the enhancer and reporter, blocking is observed in all of the lines (3 X-*scs*-*scs*' lines were reported in VAZQUEZ and SCHEDL (1994). *scs* is then challenged by placing *su(Hw)* or *Fab-8* upstream of the enhancer. In each case, blocking is retained in the majority of the transgenic lines. The photographs show eyes of flies transformed with the starting transgene XN (HAGSTROM *et al.* 1996), which has *scs*' downstream of the *white* reporter but no other boundaries and flies transformed with either the *su(Hw):scs:scs*' transgene or the *Fab-8:scs:scs*' transgene as indicated. Flies transgenic for the starting transgene, XN, have red eyes, while flies transgenic for either *su(Hw):scs:scs*' (8/9) or *Fab-8:scs:scs*' (20/21) have yellow to orange eyes as illustrated. (B) *Fab-8* in between the *white* enhancer and the *white* reporter. When *Fab-8* is interposed between the enhancer and the reporter, blocking is observed in ~80% of the lines and the eye color ranges from light yellow to orange (like that illustrated for *su(Hw):scs:scs*' and *Fab-8:scs:scs*' in A). Half of the lines retain blocking when *Fab-8* is challenged by an upstream *scs* (6/12) and have yellow to orange eye color. None of the lines (0/6) show blocking when *Fab-8* is challenged with *su(Hw)*. Photographs in B show examples of two of the nonblocking lines for *scs:Fab-8:scs*'.

As can be seen in Figure 3A, placing either *su(Hw)* or *Fab-8* in the upstream position has little effect on *scs* boundary activity, and in both cases, blocking is still observed in the vast majority of the lines. These results are consistent with all three models.

Blocking activity of an interposed Fab-8 is compromised by the upstream boundary: We next interposed the BX-C CTCF-dependent boundary *Fab-8* between the *white*

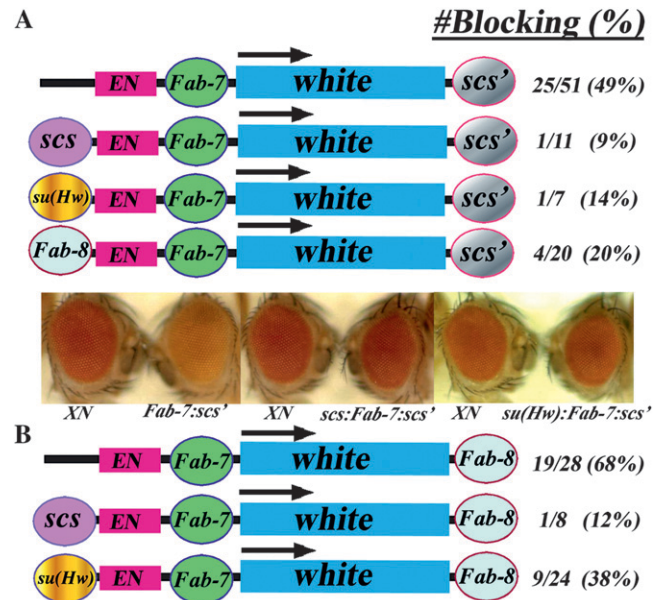


FIGURE 4.—Triple boundary with *Fab-7* in the middle. (A) *Fab-7* in between the *white* enhancer and the *white* reporter and *scs*' downstream. When *Fab-7* is interposed between the enhancer and reporter, blocking is observed in 50% of the transgenic lines (HAGSTROM *et al.* 1996); compare XN to *Fab-7:scs*' on the left. *Fab-7* is then challenged by placing *scs* (see *scs:Fab-7:scs*' in the middle), *su(Hw)* (see *su(Hw):Fab-7:scs*' on the right) or *Fab-8* (not shown) upstream of the enhancer. In all three cases, *Fab-7* blocking is compromised, and the number of transgenic lines showing blocking activity is reduced. (B) *Fab-7* in between the *white* enhancer and the *white* reporter with *Fab-8* rather *scs*' downstream of the reporter. Replacing the downstream *scs*' with *Fab-8* improves *Fab-7* blocking activity and instead of 50%, ~70% of the transgenic lines have blocking activity. When *scs* is placed upstream of the *white* enhancer, *Fab-7* blocking activity is again absent in most of the transgenic lines. While *Fab-7* blocking activity in this transgene is also reduced when *su(Hw)* is placed upstream of the *white* enhancer, the effects of *su(Hw)* are not as strong as those observed in the transgene that has *scs*' downstream of the reporter.

enhancer and reporter. When coupled with *scs*' at the downstream position, *Fab-8* is a somewhat less effective enhancer blocker than *scs* and the *white* enhancer is blocked in ~80% of lines. We then challenged *Fab-8* with either *scs* or *su(Hw)*. In the roadblock and sink models, the outcome is expected to be the same as that predicted when *scs* is challenged by an upstream *Fab-8* and *su(Hw)* boundary—blocking activity should be retained. In the loop model, blocking the intervening *Fab-8* boundary will be bypassed if the favored boundary combination is between *scs* and *scs*' as would be suggested by the results described in the previous section. Similarly, since YAMAGUCHI *et al.* (2001) reported that there are genetic interactions between BEAF and the gene encoding the Su(Hw) protein, it is possible that *scs*' will preferentially combine with *su(Hw)* rather than *Fab-8*, and *Fab-8* blocking activity will be lost.

The results of these experiments are shown in Figure 3B. When *Fab-8* is challenged by *scs*, its blocking activity is not detected in half of the lines and the eye color is comparable to that in control constructs lacking an interposed boundary. In addition, as shown in Figure S2, in the lines that still show blocking, there is a shift in the phenotypic spectrum toward a darker eye color. This would suggest that even in these lines the upstream *scs* element competes with *Fab-8* and weakens its blocking activity. The effects of an upstream *su(Hw)* element are equivalent if not more dramatic than *scs*. As indicated in Figure 3B, blocking activity is absent in all six of the *su(Hw):w_{en}:Fab8:white:scs'* lines that were recovered. These findings do not fit with the predicted behavior of boundary elements in either the roadblock or sink models. They would, however, be compatible with the predictions of a model that invokes loop formation as the mechanism of boundary function.

Fab-7 blocking activity is compromised by an upstream boundary: To further test the predictions of the roadblock, sink, and loop models, we interposed the CTCF-independent BX-C boundary, *Fab-7*, between the *white* enhancer and reporter and then challenged with an upstream boundary (see Figure 4A). When coupled with *scs'* downstream of the *white* reporter, *Fab-7* boundary activity is subject to chromosomal position effects and only about half of the lines have blocking activity. As was seen for the interposed *Fab-8* element, the introduction of a boundary at the upstream position (B3 in Figure 2) weakens *Fab-7* blocking activity. When *scs* is placed upstream, enhancer-blocking activity is only maintained in ~10% of the transgenic lines. This is close to the background frequency of *w_{en}:white:scs'* transgenic lines that have a lighter eye color phenotype. An equivalent reduction in *Fab-7* blocking activity is observed when *su(Hw)* is placed in the upstream position.

The upstream *scs* and *su(Hw)* boundaries and the downstream *scs'* boundary are derived from chromosomal DNA segments (or transposons) that have no apparent functional relationship to the homeotic genes or *cis*-regulatory domains of the BX-C. Thus, it seemed possible that the deleterious effects of the upstream boundaries on *Fab-7* and *Fab-8* blocking activity might be an anomaly arising from some sort of incompatibility between BX-C boundaries and boundaries derived from elsewhere in the fly genome. To explore this possibility, we placed the *Fab-8* boundary in the upstream position. As shown in Figure 4A, the *Fab-8* boundary functions in this context much like an upstream *scs* or *su(Hw)* element and substantially reduces the enhancer-blocking activity of the interposed *Fab-7* boundary.

To examine the compatibility question further, we replaced the *scs'* element downstream of the *white* reporter with *Fab-8*. As shown in Figure 4B, the frequency of blocking *Fab-7* transgenic lines increases to slightly more than two-thirds when *Fab-8* is downstream

of the reporter. We then introduced *scs* and *su(Hw)* upstream of the *w* enhancer. While a downstream *Fab-8* afforded little protection against *scs*, the effects of an upstream *su(Hw)* element are partially ameliorated and the frequency of lines retaining blocking activity when *Fab-8* is in the downstream position is increased compared to that observed when *scs'* was located downstream. Taken together with results described above, these findings indicate the blocking activity of a boundary element depends upon the configuration and identity of the flanking boundaries.

Domain definition: *Experimental design:* The behavior of boundaries in the triple boundary assay does not conform to the predictions of either the roadblock or sink models; instead, the changes in blocking activity induced by altering the boundary configuration would be most consistent with a mechanism involving topologically independent looped domains. To further distinguish between the three models, we devised a domain definition assay. In this assay, two reporters, *white* and *hsp70:LacZ*, flank the 4-kb upstream (UPS) and neurogenic (NE) enhancers from the *fushi tarazu* (*ftz*) gene. The UPS enhancer drives expression in seven stripes during early embryogenesis, while the NE enhancer drives expression in the embryonic CNS during midembryogenesis (HAGSTROM *et al.* 1996). As shown in Figure 5 (see also detailed map in Figure S1), the BEAF-dependent boundary *scs'* was placed downstream of the *hsp70:LacZ* reporter. When there are no other boundaries present in the transgene, the two reporters and the two *ftz* enhancers lie in the same regulatory domain. In this configuration, the *ftz* enhancers can activate both reporters; however, the NE enhancer preferentially turns on the *LacZ* reporter, presumably because the *hsp70* promoter is more compatible with the NE enhancer than the *white* promoter (see Figure 6A).

The regulatory domain is redefined when a boundary element, such as *Fab-7*, is interposed between the *ftz* enhancers and the *hsp70:LacZ* reporter (2 in Figure 5, A–C). In both the roadblock and sink models, the presence of the intervening *Fab-7* boundary places the *ftz* enhancers and the *hsp70:LacZ* reporters in different regulatory domains and blocking should be observed (see Figure 5, A2 and B2). In the loop-domain model, domain definition could be influenced by endogenous boundaries near the site of insertion; however, if the loops are defined by a combination of the B2 boundary, *Fab-7*, with *scs'* (see Figure 5, C2), blocking will be observed since the *ftz* enhancers and the *hsp70:LacZ* reporter will be in different domains. As is illustrated in Figure 6B, the robust stripe and CNS expression of β -galactosidase seen in the absence of the boundary disappears when *Fab-7* is inserted between the enhancers and the *hsp70:LacZ* reporters. On the other hand, this configuration of boundary elements places the *ftz* enhancers and the *white* reporter in the same regulatory domain. As would be predicted by all three

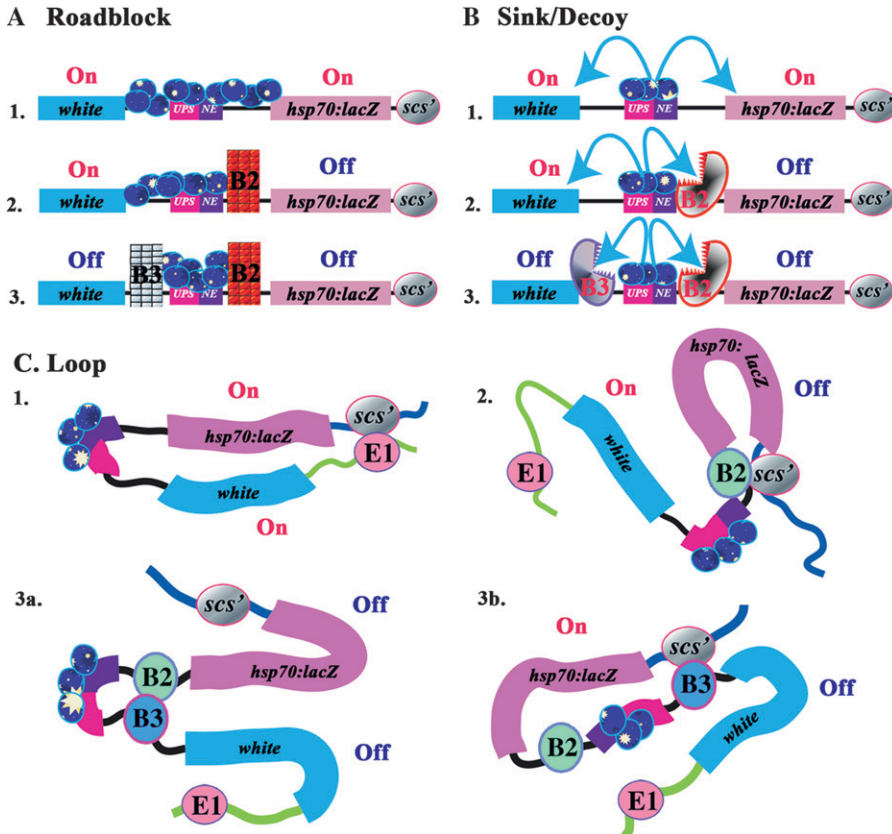


FIGURE 5.—Domain definition assay. The transgene in the domain definition assay has *white* (blue) and *hsp70:lacZ* (mauve) reporters flanking two enhancers, the UPS stripe enhancer (red) and the NE neurogenic (purple) enhancer, from the *ftz* gene, which are active during early and midembryogenesis. *scs'* is located downstream of the *hsp70:lacZ* reporter. Additional boundaries are then introduced into the transgene between the *ftz* enhancers and the *hsp70:lacZ* reporter (B2) and/or between the *ftz* enhancers and the *white* reporter (B3). As shown in A–C1, the *ftz* enhancers are expected to activate both the *white* and *hsp70:lacZ* reporters in all three models when there are no intervening boundaries (On). In (A–C2), a boundary (B2) placed between the *hsp70:lacZ* reporter and the *white* reporter (B3) is expected to generate two regulatory domains, one containing the *hsp70:lacZ* reporter and the other containing the *ftz* enhancers and the *white* reporter. In this domain configuration, the *ftz* enhancers will activate the *lacZ* (Off) but not the *white* (On) reporter in all three models. Similarly, a boundary (B3) placed between the *ftz* enhancers and *white* is expected to block activation of the *white* but not the *hsp70:lacZ* reporter in all three models (not shown).

As indicated in 3 for the roadblock (A) and sink (B) models, flanking the *ftz* enhancers with boundaries B2 and B3 will subdivide the transgene into three independent domains, containing, respectively, *white*, the *ftz* enhancers, and *hsp70:lacZ*. In the loop model, domain definition depends on the identity and relative position of the boundary elements in the transgene and in the neighboring chromosomal DNA segments. Among the many possible domain configurations, two different examples are shown for the loop model in C3a and C3b. In C3a, a loop domain is defined by B2 and B3 [interacting with each other as shown, or with some nuclear structure(s)]. This subdivides the transgene into three separate regulatory domains containing, respectively, *white*, the *ftz* enhancers, and *hsp70:lacZ*. In this case both reporters are protected from the *ftz* enhancers. In C3b, a loop domain is defined by B3 and *scs'*. This subdivides the transgene into a domain containing *white*, and a domain containing the *ftz* enhancers, boundary B2 and the *hsp70:lacZ* reporter. In this case, the *ftz* enhancers are blocked from activating *white*, but are not prevented from activating *hsp70:lacZ*.

models, *white* is expressed in a stripe pattern during early embryogenesis and in a segmentally repeating pattern in the CNS during midembryogenesis (Figure 6B). [The regulatory domain should also be redefined when a boundary is placed between *white* and the *ftz* enhancers (B3). In this case, the *ftz* enhancers will drive expression of the *hsp70:LacZ* reporter, while activation of the *white* reporter should be blocked.]

The three models for boundary function make different predictions about how regulatory domains will be defined when boundaries are interposed simultaneously between the two reporters and the *ftz* enhancers. In both the roadblock and sink models, boundaries B2 and B3 will generate three independent regulatory domains—the *white* reporter, the two *ftz* enhancers, and the *hsp70:LacZ* reporter—and neither reporter will be active [Figure 5 roadblock (A3) and sink/decoy, (B3)] In the loop model, reporter activity will be determined by which combination of boundaries is used to generate the loops. If three separate loops are

formed—the first containing *white*, the second the two *ftz* enhancers, and the third the *hsp70:LacZ* reporter—then both reporters will be off (Figure 5, C3a). On the other hand, if the loop is defined by combining boundaries B3 and *scs'* (Figure 5, C3b), the *ftz* enhancers and the *hsp70:LacZ* reporter will reside in the same looped domain (together with the unpaired boundary B2) and β -galactosidase expression will be activated. Other sorts of loop configurations and regulatory interactions are possible, depending upon the identity of boundaries B2 and B3 and the potential effects of endogenous boundaries flanking the transgene insertion site. Moreover, if the factors conferring blocking activity in early and midembryogenesis are distinct, as is the case for *Fab-7*, the domain definition pattern could change from one stage to the other. (In the roadblock and sink models, this would only happen if one of the boundaries is inactive at a specific stage or tissue.)

Challenging Fab-7 with Fab-8: In the first experiment, a *Fab-7* boundary located between the *ftz* enhancers and

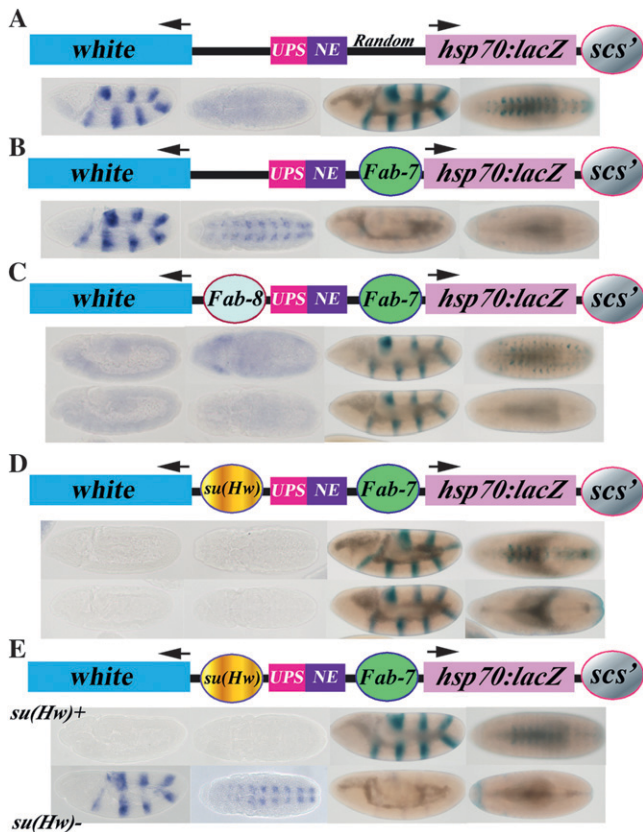


FIGURE 6.—Domain definition depends upon boundary identity and relative position. (A) In the starting transgene containing a random segment of *Drosophila* DNA (from the *Bicaudal-D* locus) or no DNA (not shown) in between the *ftz* enhancers and the *hsp70:lacZ* reporter the *ftz* enhancers activate both *white* and *hsp70:lacZ*. Lines are from HAGSTROM *et al.* (1996). Note that the NE enhancer does not activate *white* when a competing *hsp70:lacZ* reporter is accessible. (B) When *Fab-7* is placed between the *ftz* enhancers and the *hsp70:lacZ* reporter, it subdivides the transgene into two independent regulatory domains. Lines are from HAGSTROM *et al.* (1996). One domain contains *white* and the *ftz* enhancers, while the other domain contains the *hsp70:lacZ* reporter. In this configuration of regulatory domains, the *ftz* enhancers drive *white* expression but are blocked from activating *hsp70:lacZ* expression. (C) The regulatory domains in B are redefined when *Fab-8* is interposed between the *ftz* enhancers and *white*. In the first example, both *ftz* enhancers now drive *hsp70:lacZ* expression, while they no longer activate *white*. From this pattern of gene activity, we infer that the organization of regulatory domains resembles that in Figure 5, 3b. The *ftz* enhancers (along with *Fab-7*) are now in the same regulatory domain as *hsp70:lacZ*, while *white* is in a separate regulatory domain. In the second example, the *ftz* UPS enhancer activates *hsp70:lacZ*, but not *white*, while the NE enhancer activates neither of the reporters. From this pattern of gene activity we infer that the organization of regulatory domains at the time when the UPS enhancer is active resembles that in Figure 5, 3b. Later in development, when the NE enhancer is active, the organization of regulatory domains changes and it resembles that shown in Figure 5, 3a. In this case, the *ftz* NE enhancer is separated from both *white* and *hsp70:lacZ* and neither reporter is activated. (D) A similar pattern of domain redefinition is observed when *su(Hw)* is interposed between the *ftz* enhancers and *white*. In 9 of 10 transgenic lines, the *ftz* UPS enhancer activates *hsp70:lacZ*, but not

the *hsp70:lacZ* reporter was challenged by placing *Fab-8* in between the *ftz* enhancers and *white*. Figure 6C shows that the interposed *Fab-8* boundary blocks both the UPS and NE enhancers from activating *white* and in all of the lines examined both *white* stripe and CNS expression are substantially reduced. While this observation would be consistent with the expectations of the roadblock and sink models, the pattern of β -galactosidase expression is not. We found that *Fab-7* blocking of the UPS enhancer is compromised in seven out of eight transgenic lines containing the upstream *Fab-8* boundary, and as illustrated for two of the lines in Figure 6C, the UPS enhancer activates robust stripe expression. In addition, the effects of the upstream *Fab-8* boundary on *Fab-7* blocking activity are tissue and/or enhancer specific. While there is little or no blocking of the stripe enhancer in early embryos, *Fab-7* boundary activity seems to be reestablished in midembryogenesis and it is able to block the NE enhancer from activating β -galactosidase expression in the CNS in five out of the seven *Fab-8:UPS/NE:Fab-7* lines we tested. In the two remaining lines, blocking activity is compromised and the NE enhancer activates β -galactosidase expression.

Challenging Fab-7 with su(Hw): In the second experiment, *Fab-7* was challenged with the *su(Hw)* boundary. Consistent with the expectations of all three models, we found that the interposed *su(Hw)* boundary blocks the *ftz* enhancers from activating *white* in all of the transgenic lines that were isolated. On the other hand, as was observed for *Fab-8*, the effects of incorporating *su(Hw)* into the transgene are inconsistent with the predictions of both the roadblock and sink models. We found that the *Fab-7* boundary is bypassed when it is challenged by the *su(Hw)* boundary and the UPS enhancer drives β -galactosidase stripe expression in nine out of ten transgenic lines. This is illustrated for two of the lines in Figure 6D. Thus, placing *su(Hw)* between *ftz* enhancers and the *white* reporter effectively abrogates the UPS enhancer-blocking activity of the *Fab-7* boundary. The results obtained for the NE enhancer are also inconsistent with the predictions of both the roadblock and sink models. At this point in development, the boundary activity of the *Fab-7* element is insertion-site dependent. In half the lines, *Fab-7* boundary activity is disrupted by the upstream *su(Hw)* element and the NE enhancer drives β -galactosidase expression in the CNS (Figure 6D, top). In

white (D, upper and lower panels, Figure 5, 3b), while in the remaining line neither reporter is active (Figure 5, 3a). In midembryogenesis, the *Fab-7* boundary is inactive in half of the lines, and the *ftz* NE enhancer activates *hsp70:lacZ* but not *white* (D, top panel). In the remaining lines, *Fab-7* boundary activity is reestablished and neither reporter is active (D, bottom panel). (E) Domain redefinition depends upon a functional *su(Hw)* boundary. In this example, both *ftz* enhancers drive *hsp70:lacZ* expression but not *white* in wild-type flies, while in *su(Hw)* mutant flies where the *su(Hw)* boundary is inactive, the *ftz* enhancers activate *white*, but not *hsp70:lacZ*.

the other half, *Fab-7* boundary activity is apparently unperturbed, and there is a clear reduction in NE-dependent β -galactosidase expression (Figure 6D, bottom).

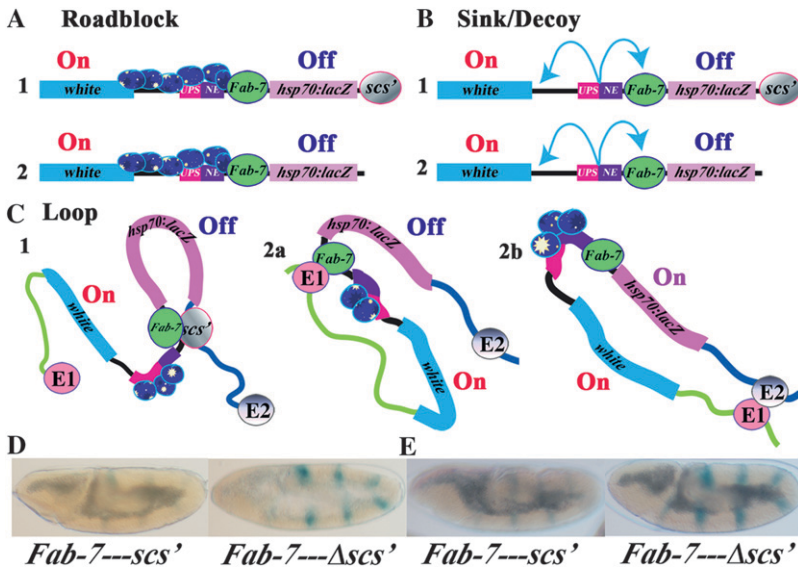
Fab-7 blocking activity is restored by mutations in *su(Hw)*: The patterns of domain definition observed when *Fab-7* is combined with *Fab-8* or *su(Hw)* are inconsistent with the predictions of both the roadblock and sink models and to account for these findings, some sort of “special” explanation has to be invoked. For example, it is possible that the three-boundary combination targets the transgene to chromosomal sites that are unfavorable for *Fab-7* activity especially in early embryos. If this were correct, then the inactivation of *Fab-7* in the domain definition reporter would not depend upon the enhancer-blocking function of the *Fab-8* or *su(Hw)* boundary. To test this possibility, we introduced three of the *su(Hw)*–*Fab-7* domain definition inserts into a *su(Hw)* mutant background. The enhancer-blocking activity of the *su(Hw)* boundary requires the Su(Hw) protein, and, as would be expected from many previous studies, we found that the UPS and NE enhancers drive *white* expression in the *su(Hw)* mutant embryos in all three lines (see example in Figure 6E). In addition to eliminating blocking by the *su(Hw)* boundary, the *su(Hw)* mutation also restores *Fab-7* boundary activity. The reestablishment of *Fab-7* blocking activity was observed in all three transgenic lines and is shown for one of the lines (in which both UPS and NE blocking activity of the *Fab-7* element is lost when *su(Hw)* boundary is functional) in Figure 6E. These findings indicate that special chromosomal position effects are unlikely to be responsible for the loss of *Fab-7* blocking activity in the domain definition experiment. They also argue that the *Fab-7* boundary in these transgenes is fully “functional” in early and midstage wild-type embryos even in the lines in which no blocking is observed.

Single or in combination? Blocking activity in the roadblock and sink models is an intrinsic characteristic of the boundary element and is entirely independent of the presence or absence of other nearby boundaries. In contrast, in the topological loop model, boundary function depends upon which boundary element combination is used to delimit the regulatory domain. For this reason, the activity of a boundary in the loop model will be context dependent and determined by the location and nature of other nearby boundaries. It should be possible to further distinguish between these models by testing whether boundaries function as single units or in combination. For this purpose, we generated a modified version of the domain definition transgene (see Figure 7, A–C1,2), which has the *Fab-7* boundary in between the *ftz* enhancers and the *hsp70:LacZ* reporter and an *scs'* element downstream of the reporter that is flanked by *ScdI* sites and can be excised *in situ*. In the roadblock and sink models, removing the downstream *scs'* boundary will expose the *hsp70:LacZ* reporter to enhancers/silencers located in the

chromosomal DNA segment adjacent to the insertion site; however, this should have no effect on *Fab-7* blocking of the *ftz* enhancer (see Figure 7, A2 and B2). In the loop model, the blocking activity of *Fab-7* following the removal of *scs'* will depend upon how well it functions in combination with endogenous boundary elements upstream (E1) or downstream (E2) of the transgene. As illustrated in Figure 7, C2a, blocking activity could be conserved if *Fab-7* is a good match for one of these boundaries; however, if the boundaries located to either side of the transgene combine preferentially with each other, and not with *Fab-7*, blocking activity will be reduced or lost (Figure 7, C2b).

We compared *Fab-7* blocking before and after *in vivo* excision of *scs'*, with *ISceI* nuclease, in two transgenic lines. As illustrated in Figure 7, D and E, *Fab-7* blocking of the UPS stripe enhancer is partially compromised in both lines when *scs'* is removed. While blocking of the NE enhancer is retained in both lines after excision of *scs'*, β -galactosidase is expressed in a nonspecific pattern in older embryos (not shown). Presumably this expression pattern is generated by regulatory elements in the chromosomal DNA segment downstream of each transgene insertion site.

Boundary resurrection: In the domain definition assay we found that *Fab-7* blocking of the *ftz* enhancers could be compromised by introducing a *Fab-8* or *su(Hw)* boundary upstream of the *ftz* enhancers. This finding is not readily explained by either the roadblock or sink/decoy model and as noted above would require some novel explanation or mechanism. One mechanism, which would fit best with the roadblock model, is that the loss of *Fab-7* blocking activity could be a consequence of confining excessive *ftz* enhancer activity between two closely spaced boundaries. In the absence of the upstream *Fab-8* or *su(Hw)* boundary, any excess enhancer activity not used to activate *white* would be dissipated by the tracking of the activator complex through the *white* gene into the adjacent chromosomal DNA. However, when the *ftz* enhancers are confined by two closely flanking boundaries, excess activity might build up until it overwhelmed the weaker boundary, (which in these experiments would be *Fab-7*) and activated the nearby reporter. (In a recast version of the sink/decoy model, boundaries would repel rather than capture the looping enhancer. In this case, when the *ftz* enhancers are flanked by two boundaries, the “weaker” boundary, *Fab-7*, would be overcome by the combination of boundary repulsion and enhancer activity.) Alternatively, it is possible that certain boundary elements are able to neutralize other nearby boundaries. This would be a “novel” activity as the properties of boundaries postulated in either the roadblock or sink/decoy model do not require or include such a deactivation function. Moreover, since the normal position dependence for boundary action (the boundary must be placed between the regulatory element and the target gene) is violated



transgene insertion site. (D and E) UPS-dependent stripe expression (*Fab-7*–*scs'*) before and after (*Fab-7*– Δ *scs'*) for two different transgenic lines. In both lines, *Fab-7* blocks the UPS enhancer in the *Fab-7*–*scs'* transgene, and there is only a low level of *hsp70:lacZ* stripe expression as seen previously for *Fab-7* (HAGSTROM *et al.* 1996). However, when *scs'* is deleted, UPS-dependent stripe expression is activated.

when the *Fab-7* boundary is neutralized, this novel activity would have to involve mechanisms distinct from those deployed when an interposed boundary blocks enhancer action.

To test these novel mechanisms, we asked whether *Fab-7* blocking activity in a domain definition transgene, which contains an upstream *Fab-8* element, can be restored by removing the *scs'* boundary downstream of the *hsp70:LacZ* reporter. If *Fab-7* blocking activity is lost because of an accumulation of excess enhancer activity, removing *scs'* would not be expected to restore *Fab-7* blocking activity. The *Fab-8* and *Fab-7* boundaries would still confine the *ftz* enhancers and excessive enhancer activity should still accumulate. Similarly, if *Fab-7* is inactivated by the upstream *Fab-8* boundary, it should still be inactivated when *scs'* is removed. In fact, the loss of blocking activity might be even more severe as the data presented in Figure 7 argue that *Fab-7* blocking activity depends upon the downstream *scs'* boundary. In contrast, in the loop model, the bypass of *Fab-7* induced by the upstream *Fab-8* or *su(Hw)* boundary occurs because these boundaries preferentially combine with *scs'* (and/or endogenous boundaries near the site of insertion) to generate the looped domain (see Figure 8, C1). As indicated in Figure 8, C2a and C2b, removal of *scs'* changes the neighborhood and this should lead to new combinatorial interactions. These combinations could restore *Fab-7* blocking activity (Figure 8, C2a). Alternatively, *Fab-7* could still be bypassed (Figure 8, C2b).

We examined β -galactosidase expression in *Fab-8:Fab-7:scs'* embryos before and after excision of the downstream *scs'* boundary and the results for two transgenic

FIGURE 7.—Single or in combination? Predictions of the barrier, sink/decoy, and loop models when the downstream *scs'* boundary is present or absent are shown in A–C. When *scs'* is present (A–C, 1) all three models predict that the *Fab-7* boundary will block the *ftz* enhancer from activating *hsp70:lacZ*. In the barrier and sink/decoy models blocking activity is an intrinsic property of a boundary. Consequently, as shown in A2 and B2, removing the downstream *scs'* boundary should have no effect on *Fab-7*, and it should still block the *ftz* enhancers from activating *hsp70:lacZ*. In contrast, in the loop model, the regulatory domains will be redefined whenever a boundary is removed or added. In the redefined domains, *Fab-7* could still block the *ftz* enhancers from activating *hsp70:lacZ* (C2a), or *Fab-7* blocking activity could be lost (C2b). In the former case, a looped domain would be defined by *Fab-7* and an endogenous boundary upstream of the transgene insertion site (E1). In the latter case, a looped domain is formed between boundaries upstream (E1) and downstream (E2) of the

lines are shown in Figure 8, D–E and F–G, respectively. In the first line, *Fab-7* blocking of the *ftz* UPS and NE enhancers is compromised by the upstream *Fab-8* boundary, and β -galactosidase is expressed in a stripe pattern during early embryogenesis and in the CNS during midembryogenesis (Figure 8D). As can be seen in Figure 8E, excision of the *scs'* boundary downstream of the *hsp70:LacZ* reporter restores *Fab-7* blocking of both the UPS and NE enhancers. In the second line, the upstream *Fab-8* boundary interferes with *Fab-7* blocking of the UPS enhancer, but does not seem to affect blocking of the NE enhancer (though there is background mesoderm/endoderm β -galactosidase expression in this line; Figure 8F). In this case, *Fab-7* blocking of the UPS enhancer is restored when *scs'* is excised. (Note also that the background β -galactosidase expression in the mesoderm/endoderm seen at later stages also seems to be somewhat enhanced when *scs'* is deleted.) Similar results were obtained for two other transgenic lines (not shown). These findings would be inconsistent with the expectations of the “revamped” versions of the roadblock and sink models discussed above, while they would fit the predictions of the loop model.

DISCUSSION

Differentiating between models for boundary element function: Several findings have linked boundary activity to the higher order organization of the chromatin fiber. The first were studies on the *Notch* locus, which identified a small deletion, *facet-strawberry* (*fa^{sub}sub*), which both altered the structural organization of the *N* locus in polytene chromosomes, fusing the *N* chromomere or

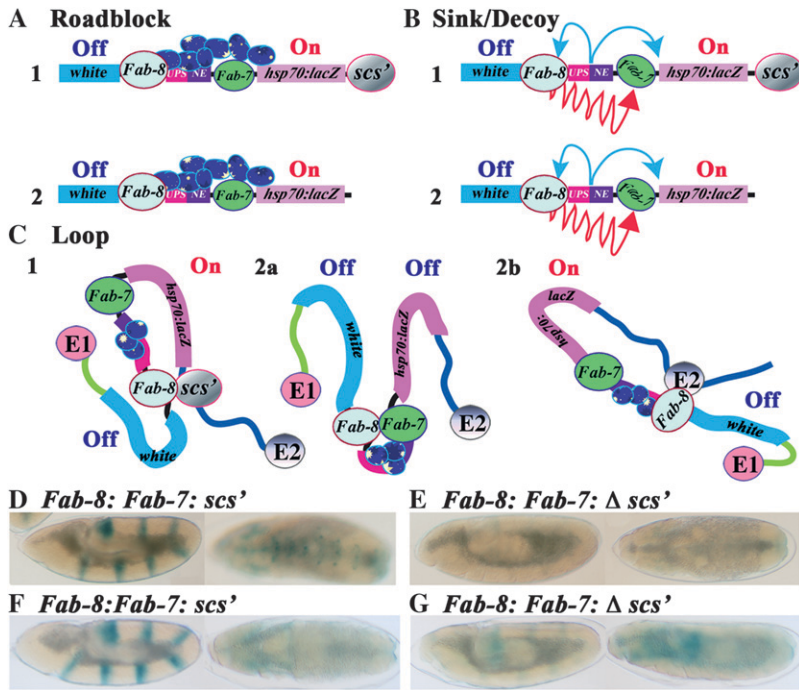


FIGURE 8.—Boundary resurrection. In the roadblock and sink/decoy novel mechanisms must be postulated to account for the loss of *Fab-7* blocking activity in the domain definition assay. One mechanism, illustrated for the roadblock model in A1, postulates that when enhancers are tightly confined by two flanking boundaries, excess enhancer activity accumulates and eventually overcomes the weaker boundary. A second mechanism, illustrated for the sink/decoy model in B1, postulates that certain boundaries have a novel ability to neutralize or inactivate (red arrows) other nearby boundaries. A2 and B2 show that the special mechanisms postulated to cause the loss of *Fab-7* blocking activity in the barrier and sink/decoy models (confining the *ftz* enhancers or boundary inactivation) should be completely indifferent to the presence or absence of a downstream *scs'* element. In contrast, the loop model predicts that the configuration of regulatory domains will be redefined whenever a boundary is removed. In C2a, *Fab-7* and *Fab-8* define a new domain that contains the *ftz* enhancers. In this domain configuration, *Fab-7* boundary activity will be restored. In C2b, the new domain is formed by *Fab-8* and an endogenous boundary (E2) downstream of

the transgene insertion site. In this configuration, the *ftz* enhancers, *Fab-7* and *hsp70:lacZ*, are in the same regulatory domain, and the reporter will be activated. (D–G) UPS- and NE-dependent *hsp70:lacZ* activity before (D and F) and after (E and G) the removal of *scs'* for two different transgenic lines.

band 3C7, with the adjacent band, 3C6, and induced a chromosomal position effect that downregulated *N* activity (KEPPY and WELSHONS 1977; RYKOWSKI *et al.* 1988). Like the boundaries described here, the *fa^{sub}* sequence functions as a boundary element in transgenic reporter assays (VAZQUEZ and SCHEDL 2000). The 3C experiments have also shown that the differential regulation of the imprinted *H19/Igf2* locus in mouse correlates with the formation of CTCF-dependent parent-of-origin specific loops (MURRELL *et al.* 2004; ENGEL *et al.* 2008; NATIVIO *et al.* 2009). Similarly, the boundaries flanking the mouse β -globin locus pair with each other to generate a loop domain containing the globin genes and the locus control region (SPLINTER *et al.* 2006). In addition, insulator-mediated chromosomal loops have been directly visualized in nuclear halos produced from *Drosophila* nuclei (BYRD and CORCES 2003). It was shown that the protein components of the *su(Hw)* insulator, Su(Hw) and Mod(mdg4), cofractionate with the nuclear matrix and reside at the bases of loops of DNA. Intriguingly, adding an insulator in the middle of the loop, reorganized the looped domain into two smaller loops, anchored to the nuclear matrix (BYRD and CORCES 2003).

Though these and other observations support the idea that boundaries subdivide the chromosome into a series of topologically independent loops, it has generally been assumed that their insulating mechanism will not depend upon this topological isolation, but rather

upon how enhancers/silencers act at distance. Current models for enhancer action invoke either a tracking or a looping mechanism (BLACKWOOD and KADONAGA 1998), and in each model boundaries are expected to have rather different properties. In the tracking model, boundaries function passively as roadblocks, preventing regulatory complexes from moving processively along the chromatin fiber toward the promoter. By contrast, in the looping model, boundaries act as sinks or decoys and must actively capture the looping enhancer/silencer before it can make contact with the target gene. While the roadblock and sink/decoy models differ substantially in mechanistic detail, they are similar in that they both presuppose that insulating activity is an intrinsic property of the boundary element and is entirely independent of the nature and location of other nearby boundaries. This is not the case with the topological loop model. Since two boundaries are needed to define the endpoints of the looped domain, this model requires that boundaries function in combination. Because some combinations will necessarily be better than others, the configuration of looped domains, and consequently the insulating activity of a particular boundary, will depend upon the identity and relative location of boundaries in the region of interest. The properties of boundary elements postulated by these three models are distinct and each model makes quite different predictions about how these elements will behave in different experimental contexts. In the studies reported

here, we have designed several assays that test whether the attributes of boundary elements conform to those predicted by the three models.

The first, the triple boundary assay (Figure 2), tested for boundary competition. In this assay, a boundary interposed between a *white* enhancer and a *white* reporter was challenged by introducing another boundary upstream of the enhancer. We tested five different boundaries placed in different combinations at sites downstream of the reporter, in between the enhancer and reporter, and upstream of the enhancer. Depending upon the combination and configuration of the three boundaries in the transgene, we found that blocking activity was unaffected, weakened, or lost altogether. These findings indicate that boundaries can compete with each other and would only be consistent with the predictions of the topological loop model.

In the domain definition assay (Figure 5), we used two reporters, *white* and *hsp70:LacZ*, flanking a pair of *ftz* enhancers to explore how boundary elements define regulatory domains. Consistent with all three models, when *Fab-7* is placed between the *ftz* enhancers and the *hsp70:lacZ* reporter, the transgene is subdivided into a domain containing the *ftz* enhancers and *white* and a domain containing just the *lacZ* reporter. However, when an *su(Hw)* or *Fab-8* boundary is then placed between *white* and the *ftz* enhancers, in most instances two not three regulatory domains are generated, one containing *white* and the other containing the *ftz* enhancers, the *hsp70:lacZ* reporter and a bypassed *Fab-7* boundary. These results fit the predictions of the topological loop model, but not the roadblock or sink model. Moreover, consistent with a strong prediction of the loop model, the bypass of *Fab-7* requires a functional *su(Hw)* boundary. When the *su(Hw)* boundary is inactivated by mutations in the *su(Hw)* gene, the regulatory domains in the transgene are redefined and *Fab-7* blocking activity is restored.

The third assay tested whether boundaries function singly or in combination. In this experiment, we asked whether *Fab-7* blocking of the *ftz* enhancers in the domain definition transgene depends upon the *scs'* boundary located downstream of the *hsp70:LacZ* reporter. We found excising the *scs'* boundary can compromise *Fab-7* blocking activity. This result is consistent with expectations of the topological loop model and supports the idea that enhancer blocking requires boundaries to function in combination.

Finally we asked whether the disruption of *Fab-7* blocking activity when *Fab-8* is placed upstream of the *ftz* enhancers in the domain definition transgene can be rescued by removing the downstream *scs'* boundary. In the topological loop model, context is critical for boundary function and for that reason eliminating one of the competing boundaries might be expected to resurrect blocking activity. Indeed this was the case. We found that excising *scs'* can reestablish *Fab-7* blocking activity.

Of course, it is possible to envision other novel explanations for why the behavior of boundaries in these different assays does not conform to the predictions of the roadblock or sink/decoy models. For example, since *Fab-7* boundary activity in the domain definition transgene can be reestablished by removing the downstream *scs'* boundary, it could be argued that neutralization activity is only manifested when the target boundary is bracketed by boundary elements. However, since *scs* can be placed in the middle position in the triple boundary transgene without being inactivated, this rule of three cannot always apply. On a more general level, introducing novel explanations such as a spill over of excess enhancer activity or boundary inactivation to account for results that do not fit the predictions of the roadblock or sink/decoy model is unsatisfying as these *ad hoc* activities are not intrinsic to the proposed mechanism of insulator action in either model.

Unlike either the roadblock or sink/decoy model, the topological loop model can readily account for all of the properties of boundaries evident in our assays. Other studies also lend support to the idea that insulators function by topological isolation. Using a transient transfection assay, AMERES *et al.* (2005) generated two topologically independent loops in a plasmid containing a reporter and two sets of TetR binding sites flanking an SV40 enhancer. Loop formation was induced by expressing TetR proteins that can oligomerize and they found that this was sufficient to prevent the SV40 enhancer from activating the reporter. Similarly, *in vitro* experiments have shown that the formation of a looped domain that topologically isolates the NtrC-dependent enhancer from the *Escherichia coli glnAp2* promoter in a closed supercoiled plasmid is sufficient to disrupt transcriptional activation (BONDARENKO *et al.* 2003). Further support for the idea that boundaries function by subdividing the chromosome into topologically independent looped domains comes from studies on how insulators affect FLP-mediated recombination (KRIVEGA *et al.* 2010). These authors found that placing a single *su(Hw)* boundary in between two FRT sites substantially suppresses FLP-mediated recombination. On the other hand, when two (appropriately oriented) *su(Hw)* boundaries are interposed between the FRT sites, FLP-mediated recombination is enhanced. The authors propose that pairing interactions between the two *su(Hw)* elements (see below) loops out the intervening DNA and brings the two FRT sites into closer proximity with each other.

Implications for boundary function: *Mechanism of loop formation:* A number of different mechanisms for loop formation have been suggested. These include the association of boundaries with the nuclear matrix or some other supporting and ubiquitous nuclear structure, the recruitment of boundaries into specialized boundary-specific structures such as the insulator bodies, and direct boundary:boundary interactions. There is evi-

dence in the literature supporting each of these mechanisms. For example, *su(Hw)* boundaries are found associated with the nuclear matrix (NABIROCHKIN *et al.* 1998) and have also been shown to physically associate with each other in insulator bodies (GERASIMOVA *et al.* 2000; BYRD and CORCES 2003). This is also true for CTCF insulators (MURRELL *et al.* 2004; YUSUFZAI *et al.* 2004; SPLINTER *et al.* 2006; NATIVIO *et al.* 2009).

Our assays were not designed to distinguish between the different mechanisms of loop formation; however, the competition between boundary elements evident in the triple boundary and domain definition assays is most easily explained by direct boundary:boundary interactions or by interactions that are mediated by boundary-specific insulator bodies rather than for binding to sites in the matrix. If boundaries were competing for the same sites in the nuclear matrix or some other ubiquitous nuclear structure, then the competition should follow a consistent hierarchical pattern that reflects the relative affinity of the different boundaries for this structure. This does not seem to be the case. In the triple boundary assay, for example, an upstream *scs* boundary is less effective in competing with *Fab-8* in the blocking position than *su(Hw)*. Accordingly, *su(Hw)* would be expected to have a higher affinity for the matrix/scaffold than *scs* and this should be reflected in the relative activity of these elements in other experiments. However, an upstream *su(Hw)* does not compete with *scs* in the blocking position and is less effective in disrupting blocking than *scs* when competing against a *Fab-7* boundary in the blocking position.

Further support for the idea that specific interactions rather than an association with a ubiquitous structure are responsible for loop formation comes from studies on the “pairing-dependent” bypass of boundaries. This phenomenon was first observed for *su(Hw)* (CAI and SHEN 2001; MURAVYOVA *et al.* 2001). While a single *su(Hw)* element interposed between an enhancer and a promoter blocks activation, blocking is lost when there are two *su(Hw)* elements. Subsequently, many other boundaries have been found to exhibit pairing-dependent bypass; however, bypass appears to require some type of specific interactions as it is largely restricted to homologous boundary pairs (KUHN *et al.* 2003; GRUZDEVA *et al.* 2005; KYRCHANOVA *et al.* 2007) or to boundaries like *su(Hw)* and *IA2* that depend upon the same set of protein factors (MAKSIMENKO *et al.* 2008). Consistent with this conclusion, KYRCHANOVA *et al.* (2008) found that bypass is observed for homologous pairs of multimerized *Zw5*, CTCF, and *su(hw)* binding sites, but is not observed for heterologous multimer pairs. This specificity for homologous multimer pairs would not be readily explained by interactions of the different boundary proteins with the same ubiquitous matrix/scaffold. With the possible caveat that the boundary bypass phenomenon could reflect some function of boundary elements (such as mediating pairing

between sister chromosomes) not directly related to loop formation, these findings would also argue that the protein complexes associated with boundaries must be interacting directly with each other or interacting with their own unique set of cofactors.

Pairwise or multipartite? The effects of adding and removing boundaries from the transgenes in our assays are mostly easily understood if loop formation tends to involve pairwise boundary combinations. If simultaneous multipartite combinations between the boundaries in the transgenes were possible, then the configuration of regulatory domains (and the pattern of reporter expression) would likely have resembled that predicted in the roadblock model. For example, in the triple boundary transgene with *su(Hw)* upstream and *Fab-8* in the blocking position, a tripartite combination of all three boundaries in the transgene (either through interactions with each other or via recruitment to insulator bodies) should have generated two regulatory domains, one containing the *white* enhancer bordered by *su(Hw)* and *Fab-8* and another containing the *white* reporter bordered by *Fab-8* and *scs'*. The finding that a single interposed boundary element has blocking activity, while two copies are bypassed would also argue that boundary interactions must occur in pairs (CAI and SHEN 2001; MURAVYOVA *et al.* 2001). This view is further supported by the finding that blocking activity is restored when a third boundary is added (KUHN *et al.* 2003). On the other hand, an important caveat in all of our experiments is that the distances between the boundaries in the transgenes are rather short, ranging from ~1 kb to 5 kb. For this reason, it is possible that a less than optimal loop size or steric hindrance makes multipartite interactions between these nearby boundaries unfavorable. Other unanticipated factors could also come into play as the loop size goes from small to large. Clearly, further studies will be required to determine whether our transgene design fortuitously emphasizes boundary competition rather than collaboration.

A boundary combination code? The studies on pairing-dependent boundary bypass taken together with our findings that the blocking activity of a boundary element depends upon the identity and location of its neighbors would support the view that specific interactions determine how each boundary combines with other boundaries to define looped domains. If boundaries form loops through direct boundary:boundary interactions, then the proteins associated with each boundary could play a key role in determining its partner preference. A possible example of direct protein:protein interactions would be between the *scs* associated *Zw5* and the *scs'* associated BEAF (BLANTON *et al.* 2003). If on the other hand, boundaries function by association with specific insulator bodies, partner choice might depend upon interactions of the DNA binding proteins with bridging intermediates. For example, CTCF and Su(Hw) are reported to interact

with CP190 (PAI *et al.* 2004; MOHAN *et al.* 2007). In both of these cases, boundary activity in a given context will depend upon the availability of nearby compatible partners. While many of the boundaries identified in *Drosophila* would appear to be rather promiscuous in their ability to participate in functional combinations, it is likely that boundary activity of some elements has either been “underestimated” or missed altogether because less than optimal partners were used. For example, a full-length 1.2-kb *Fab-7* boundary blocks the *white* enhancer in only about half the lines when matched with *scs'* downstream of the *white* gene; however, blocking enhancer activity in the eye can be substantially augmented by multimerizing a small 200-bp fragment from the distal half of the *Fab-7* boundary (SCHWEINSBERG and SCHEDL 2004). A plausible explanation for the improved blocking activity is that the factors that interact with the multimer are a better match for *scs'* than the factors bound elsewhere in *Fab-7* and in the multimer these specific factors are present in multiple copies instead of only once. Similarly, we have not detected any boundary activity for the *wari* insulator at the 3' end of the *white* gene in any of our experiments (*e.g.*, KELLUM and SCHEDL 1991; GOHL *et al.* 2008); however, insulation activity is observed when *wari* is assayed with appropriate partners or transgene configurations (CHETVERINA *et al.* 2008).

Is proximity important? So far, more than eight proteins have been implicated in boundary function in flies, and it is reasonable to expect that at least several additional proteins will be identified. While there may be a sufficient number of fly boundary proteins to generate a combinatorial code that could be used to help guide preferential boundary combinations, it is not clear whether this is true in vertebrates as fewer proteins have as yet been implicated in boundary function. It is also possible that partner choice is dictated by relative proximity. Supporting this possibility is the finding that *scs'* is more successful in competing with *Fab-8* in the triple boundary assay when it is located closer to *scs'*.

Modulation of regulatory domains: Intriguingly, stage/tissue-specific effects on *Fab-7* blocking activity are evident in the domain definition transgene. In the absence of a challenging boundary, *Fab-7* blocks both the *ftz* UPS stripe enhancer and the NE CNS enhancer. When challenged by either *Fab-8* or *su(Hw)* boundary, *Fab-7* blocking of the UPS enhancer is almost always disrupted. However, this is not true for the blocking of the NE enhancer. In most of the *Fab-8* lines, *Fab-7* blocking of the NE enhancer is maintained, while it is lost in only half of the *su(Hw)* lines. *Fab-7* is not the only example of a boundary that exhibits a chromosomal context-dependent developmental regulation of boundary function. When the *Fab-7* boundary at its endogenous location in BX-C is replaced by *su(Hw)*, the boundary activity of *su(Hw)* becomes stage and tissue dependent. It is active in the ectoderm of early embryos

and in adults, but is not active in the CNS of older embryos (HOGGA *et al.* 2001). Since a CNS-specific inactivation of *su(Hw)* blocking activity is not evident when this boundary is tested in other chromosomal contexts with different enhancers/reporters (*e.g.*, HAGSTROM *et al.* 1996), this effect seems to be specific to insertion into BX-C.

In the case of *Fab-7*, the stage/tissue-specific alterations in boundary activity likely arise because different *cis*-acting sequences and *trans*-acting factors are important for boundary activity at different points in development (SCHWEINSBERG and SCHEDL 2004). Blocking of the UPS enhancer is mediated by a multi-protein complex, Elba, which is present in 0- to 6-hr embryos (AOKI *et al.* 2008; T. AOKI, unpublished data). Elba is not, however, found in older embryos and factors that are expressed at later stages and bind elsewhere in *Fab-7* are responsible for blocking the NE enhancer in the CNS. Our results would argue that Elba activity is much more sensitive to competition by *Fab-8* or *su(Hw)* than the factors that confer *Fab-7* blocking activity later in development. A similar explanation is not likely to apply to the *su(Hw)* element inserted into BX-C as the proteins known to be important for *su(Hw)* boundary activity are expressed ubiquitously. The loss of *su(Hw)* insulator activity in the CNS could be due to tissue-specific changes in the factors associated with the nearby BX-C boundaries, which make them less compatible with *su(Hw)* insulator. It could also reflect a tissue and chromosomal locus-specific modulation in Su(Hw) DNA binding activity. This possibility is suggested by the differences in the chromosomal distribution of DNA binding boundary proteins like Su(Hw) that are evident in different tissue culture cell lines (BUSHEY *et al.* 2009). Additionally, it is known that boundary proteins are subject to a variety of post-translational modifications that could influence their activity (YU *et al.* 2004; CAPELSON and CORCES 2005, 2006). However, whatever the mechanism, the redefinition of regulatory domains seen in our experiments [and with *su(Hw)* inserted into BX-C] raises the possibility that changing the domain organization of the chromosome is used as a mechanism for regulating gene activity during development.

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Supporting Information

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Mechanism of Chromosomal Boundary Action: Roadblock, Sink, or Loop?

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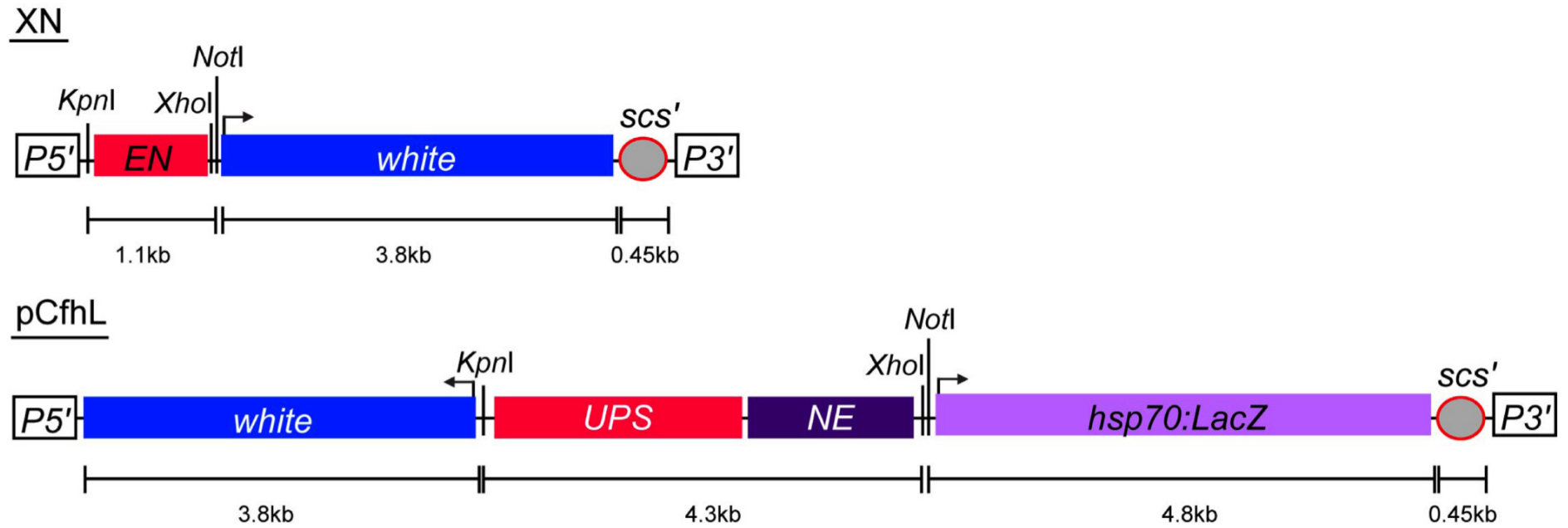


FIGURE S1.—Maps of the XN and pCfhL reporter transgenes. Boundary elements were cloned into the *Kpn*I and *Xho*I, *Not*I sites of these two vectors. The sizes of the enhancer and reporter fragments used are indicated below the maps.

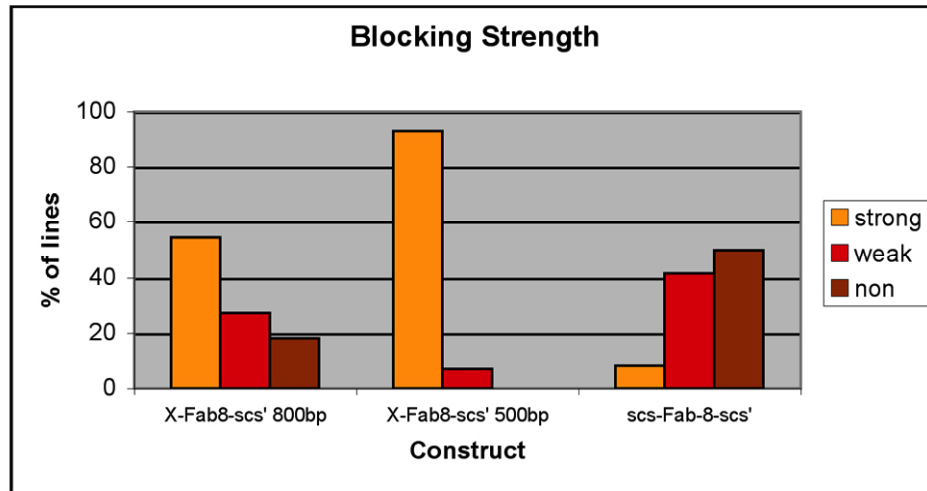


FIGURE S2.—Shift in phenotypic spectrum. Phenotypic spectrum of transgenic lines is shown for two constructs that have different size (800 bp and 500 bp) *Fab-8* fragments interposed between the *white* enhancer and the *white* reporter and the *Fab-8* construct that has *scs* located upstream of the *white* enhancer.

As can be seen in this Figure, when *scs* is placed upstream of the *white* enhancer, *Fab-8* blocking activity is fully compromised in about half of the lines. In most of the remaining lines, the upstream *scs* element appeared to partially disrupt *Fab-8* blocking activity, and these lines had darker eye colors than lines with only *Fab-8*. It is interesting to note that the effects were uniform across the eye, and there was no evidence of the sort of mosaic patterns that are observed in heterochromatic or Polycomb group silencing. This observation suggests that the organization of regulatory domains in these particular transgenes switches from one state (*white* enhancer blocked) to another (*white* enhancer unblocked). Moreover this must occur sufficiently often to effectively equalize *white* expression in all ommatidia. Previous studies on the *cis*-acting sequences required for *scs* blocking activity also suggest that domains are dynamic, not static. We found that it was possible to reconstitute boundary function by multimerizing small sub-fragments from *scs* [50]. Though blocking of the *white* enhancer was observed with the sub-fragments, they were often less effective than the intact *scs* element. Importantly, instead of a salt and pepper effect in the eye (in which blocking was observed in one group of cells, but not in another), the eyes were uniformly darker. The effects of weakening boundary activity on eye color suggested that there must be a dynamic interconversion between a blocking and non-blocking regulatory configuration. A similar dynamic interconversion between regulatory configurations is suggested by the experiments reported here.