Initiator Elements Function to Determine the Activity State of BX-C Enhancers

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Abstract

A >300 kb cis-regulatory region is required for the proper expression of the three bithorax complex (BX-C) homeotic genes. Based on genetic and transgenic analysis, a model has been proposed in which the numerous BX-C cis-regulatory elements are spatially restricted through the activation or repression of parasegment-specific chromatin domains. Particular early embryonic enhancers, called initiators, have been proposed to control this complex process. Here, in order to better understand the process of domain activation, we have undertaken a systematic in situ dissection of the iab-6 cis-regulatory domain using a new method, called InSIRT. Using this method, we create and genetically characterize mutations affecting iab-6 function, including mutations specifically modifying the iab-6 initiator. Through our mutagenesis of the iab-6 initiator, we provide strong evidence that initiators function not to directly control homeotic gene expression but rather as domain control centers to determine the activity state of the enhancers and silencers within a cis-regulatory domain.

Introduction

The Drosophila bithorax complex (BX-C) is one of two homeotic gene clusters in the fly and is responsible for determining the segmental identity of the posterior thoracic and each of the fly abdominal segments [1,2]. It does this by using a segmental identity of the posterior thoracic segment and each of the gene clusters in the fly and is responsible for determining the cis- regulatory region to control the parasegement-specific expression of the three BX-C homeotic genes: Ubx, abd-A and Abd-B [for review, see [3]].

Through the early genetic analysis of the BX-C, it was shown that its cis-regulatory sequences can be divided into nine parasegment-specific chromosomal domains (abx/bs, bxd/pbs, and iab-2 through iab-8), where each domain controls the activation of one of the three BX-C homeotic genes in a pattern appropriate for that parasegment [4–8]. Since their identification, these domains have been dissected using transgenic reporter assays to identify individual regulatory elements capable of modifying reporter gene expression. Among the elements identified were early embryonic enhancers (initiators), cell-type-specific enhancers, silencers and insulators [9–22]. Interestingly, although homeotic gene expression is restricted along the antero-postero (A–P) axis, many of the elements identified by transgenic analysis do not control reporter gene expression in an A–P restricted manner. These findings, when combined with the early genetic data suggest a model in which the cis-regulatory elements of the BX-C are controlled through the activation or repression of parasegment-specific chromatin domains [4,23–24].

According to this model, the BX-C functions through multiple layers of control. First, there are the enhancers that directly activate homeotic gene expression in a pattern appropriate for a specific parasegment. Based on the genetic data, these enhancers are known to be grouped in a way where all the enhancers required to produce a PS-specific pattern of homeotic gene expression are clustered into domains within the BX-C sequence. However, although these enhancers produce a pattern of homeotic gene expression appropriate for a specific parasegment, in transgenic assays, they are not restricted along the A–P axis, and are only restricted to specific cell-types [9,12,22,25].

The second layer of control comes from Polycomb-response element silencers (PREs). These silencers are thought to turn off the clusters of enhancers in parasegments where they are not needed, via modification of the local chromatin structure around the enhancers [for reviews [26–28]]. Once again, however, like the cell-type-specific enhancers, by themselves, PREs do not seem to sense positional information and can silence genes regardless of A–P position [29].

Domain boundary elements form a third layer of BX-C control. Each of the PS-specific enhancer clusters seems to be flanked by boundary elements, required to keep each cluster separate and autonomous from other clusters. In situ, loss of a domain boundary causes the fusion of PS-specific domains, resulting in mutant phenotypes, where the affected segments displays phenotypes characteristic of the other [18,22,30–31] [for review see [32]]. In transgenic assays, these elements have been shown to behave as insulators, blocking both positive and negative effects of cis-regulatory elements on reporter gene activity [13–14,17–18,21]. However, the presence of boundary elements cannot explain the A–P restriction of the BX-C regulatory elements, for, as with the enhancers and silencers, when taken out of the BX-C, boundary elements do not seem to have an A–P restricted activity.

So then, how do non-restricted regulatory elements control homeotic gene expression in an A–P position-dependent manner?
Author Summary

Understanding how genes become activated is one of the primary areas of research in modern biology. In order to decipher the DNA components required for this process, scientists have traditionally turned to transgenic reporter assays, where DNA elements are removed from their native environment and placed next to a simplified reporter gene to monitor transcriptional activation. Although this approach is powerful, it can result in artifacts stemming from the channelization of regulatory element activities into predetermined classes. In this manuscript, we investigate the biological role of elements from the Drosophila bithorax complex, called initiators. In transgenic assays, these elements have been categorized as enhancers. However, genetic analysis suggests that, in situ, these elements perform a far more complex function. Here, using a new method to repeatedly target a genetic locus for mutagenesis, we show that initiators function as control elements that coordinate the activity of nearby enhancers and silencers. Overall, our study highlights how gene expression can be controlled through a hierarchical arrangement of cis-regulatory elements.

If we are to assume that the reporter gene assays represent a reasonable estimate of the activity of the various regulatory elements in a domain, then there must be other regions in each domain that coordinates the activity of these elements across the A–P axis. Special early embryonic enhancers, called initiators, are the prime candidates to perform this function [9–12,17–18] [20,22]. As mentioned above, the activity of most of the elements isolated from the BX-C is not restricted along the A-P axis. In this respect, however, initiator elements are exceptional. In transgenic assays, these elements behave as early embryonic enhancers that activate reporter gene expression in a pattern along the A-P axis, consistent with the activity of the domain from which it was isolated. For example, the initiator identified from the iab-5 domain, which controls Abd-B expression in PS10/A5, activates reporter gene expression in PS10/A5 and more posterior segments in a pair-rule fashion [12]. Because similar elements were found in many PS-specific domains and these elements were the only elements discovered in the BX-C capable of reading the early parasegmental address set up by the maternal, gap and pair-rule gene products, it was hypothesized that initiators would act as the primary switches to determine if a domain was active or silenced. Unfortunately, although initiators are thought to play such a key role in BX-C gene regulation, their actual role has never been directly tested in vivo due to the lack of appropriate mutations and the difficulties in performing homologous recombination in Drosophila.

Thus, in order to explore the function of initiators and other regulatory elements in vivo, we developed a method to streamline the homologous recombination process for rapid, precise, and systematic mutagenesis. Using this method, called InSIRT (In Situ Integration for Repeated Targeting), we have created twenty new mutations in the iab-6 region of the BX-C, including mutations that directly test the role of the initiator in BX-C gene regulation (Figure 1 and Table 1).

Results/Discussion

Creation of an attP integration site in the BX-C

To study the cis-regulatory elements regulating BX-C homeotic gene expression within their natural chromosomal environment, we sought to design a method that could be used to rapidly and repeatedly target the BX-C for site-specific mutagenesis. Because this method is related to the SIRT method [33], we named it InSIRT for In Situ Integration for Repeated Targeting. Figure 2 provides a rough schematic of this method as used here in the BX-C. In short, homologous recombination is used to replace a genomic region of interest with an entry site (attP) recognized by the ΦC31-bacteriophage integrase [34–36]. Once a region of the genome is replaced by an attP site, a DNA fragment corresponding to the deleted region can be systematically mutagenized in vitro and reinserted into its normal chromosomal location by ΦC31 integration. As ΦC31 integration is a relatively fast process (by genetic standards), InSIRT allows site-specific mutagenesis of actual genes to be accomplished within the timeframe required to create a simple transgenic fly. For our experiments, we replaced a 19.3 kb region of the BX-C, roughly corresponding to the iab-6 cis-regulatory domain, with a 255bp ΦC31 integrase attP site (Figure 1 and Figure 2; note that the previously identified IAB5 initiator fragment [12] and the Fab-7 boundary element [30–31] are left intact by the deletion, while the area presumed to be the Fab-6 boundary is removed [22]).

Removal of cis-regulatory domains in the BX-C typically results in the homeotic transformations of posterior segments towards anterior segments. The segments transformed depend on the cis-regulatory domains removed and are transformed towards the last more-anterior segment whose cis-regulatory domain is intact. For example, deletion of the iab-6 cis-regulatory domain should result in the transformation of segment A6 (whose development is specified by iab-6) into A5 (whose development is controlled by iab-5). As we were attempting to delete only iab-6 in our deletion, we thus expected flies homozygous for our 19.3 kb deletion to display a typical iab-6 mutant phenotype. However, this was not the case. Flies homozygous for our 19.3 kb deletion have both their A5 and A6 segments transformed towards A4, indicating that both iab-5 and iab-6 activity are affected by our deletion (Figure 3). This can be clearly seen on the adult cuticle. Most of the segments of the adult fly abdomen can be identified independent of their position, by distinct cuticular features. For example, the wild type male sixth segment (A6) is distinguished from other segments by having a darkly pigmented tergite, covered in a distinctive pattern of tiny hairs, called trichomes, and is devoid of sternite bristles. Meanwhile, the fifth segment (A5) displays a similar darkly pigmented tergite that is uniformly covered with trichomes, and has a sternite with numerous bristles (Figure 3A). Flies homozygous for our deletion display an A4-like pigmentation pattern on both the male fifth and sixth abdominal tergites (Figure 3C). Also, the A6 sternite, normally devoid of bristles, displays numerous bristle like the A4 or A5 sternite. Based on these phenotypes, we named our deletion iab-5,6CI.

Although the adult cuticular phenotypes indicate that iab-5 function is affected in iab-5,6CI, this inactivation is incomplete and only some PS10/A5 phenotypes are affected. For example, in the embryonic CNS, the PS10/A5 Abd-B expression pattern is normal, indicating that in embryos, iab-5 is still active (compare Figure 3B with Figure 3D). Also, while iab-5 null mutants are sterile, iab-5,6CI mutants are fertile. Based on these results, we believe that iab-5,6CI removes an adult cuticle enhancer from iab-5, while leaving the rest of the iab-5 cis-regulatory domain intact. iab-6 function, as would be expected of the deletion we created, seems to be universally affected, as both the adult cuticle, and the embryonic CNS staining are affected (Figure 3G, 3D).

As a control for the InSIRT method, we first decided to reintegrate the 19.3 kb fragment removed in iab-5,6CI. As expected, the reintegrated line, iab-5,6CRI, reverts all phenotypes...
associated with \textit{iab-6}^{CI} and demonstrates the feasibility of our approach (Figure 3E, 3F).

To begin our dissection of the cis-regulatory elements in the \textit{iab-6} domain, we created a series of overlapping deletions spanning the 19.3 kb \textit{iab-5,6}^{CI} region (Figure 1 and Table 1) and examined their resulting phenotypes on the adult cuticle and embryonic CNS. We will first discuss deletions affecting the \textit{iab-6} initiator.

The \textit{iab-6} initiator is necessary, but not sufficient to control \textit{Abd-B} expression in PS11/A6

Previously, we identified a 2.8 kb element from \textit{iab-6} that displayed the characteristics of an initiator in a transgenic reporter assay. Accordingly, this 2.8 kb fragment was shown to be able to drive the early expression of a \textit{lacZ} reporter in a spatially restricted, pair-rule pattern, from PS11/A6 [22]. Unfortunately, as with
also be seen in the embryonic CNS, where the pattern in PS11/A6 is replaced by the pattern normally found in A5. The transformation can be seen on the adult cuticle where the 6th sternite takes on the shape normally observed in A5/A6. The trichome pattern occasionally shows signs of weak transformation towards A6. This effect [29,57] due to the loss of PRE sequences. (***) Mutations and deficiencies.

In critically examining the trichome pattern on the transformed A6. While the 2.8 kb fragment is the molecularly identified initiator from the A6-specific pattern is replaced by the pattern normally found in A5/A6 (Figure 4F). Based on these results, we conclude that this 927 bp fragment is absolutely necessary for *iab-6* activation in A6/PS11. The fact that a deletion of the initiator is capable of completely removing *iab-6* activity in the epidermis and the CNS, is consistent with the idea that the initiator functions as a switch to turn on all of the regulatory elements in a cis-regulatory domain. However, these results would also be consistent with the initiator being the sole positive regulatory element in A6/PS11. To rule out this possibility, we created another mutation that removes much of the *iab-6* cis-regulatory region but leaves intact the 2.8 kb initiator fragment. This mutation, called *iab-6* (Figure 1), also shows a strong loss of *iab-6* function (Figure 4G). Thus, although the *iab-6* initiator is critical for *iab-6* function, it is not sufficient for *iab-6* activity.

One important point to note regarding *iab-6* is that the loss-of-function (LOF) phenotype is slightly weaker than in *iab-6*. The difference between these two mutants can be seen when examining the trichome pattern on the transformed A6. While the *iab-6* mutation displays a transformed A6 with an A5-like trichome pattern (uniformly covered, Figure 4E), the *iab-6* trichome pattern still resembles that of a wild-type A6 (Figure 4F).

This suggests that although *iab-6* removes most of the *iab-6* sequence, there is still some functionality left in the domain. This is an important finding because it supports a prediction of the domain model, which suggests that removal of cell-type specific enhancers would affect individual (or grouped) characteristic, while removal of initiator elements would affect all characteristics. To test this idea more directly, we next performed an initiator swapping experiment.

### Initiators act as domain control centers for cis-regulatory domains

The domain model suggests that initiators act as switches to turn on (or off) the various regulatory elements present in a domain. In the simplest state, this would mean that initiators would not participate directly in driving homeotic gene expression, but would simply coordinate the activity of cell/tissue-type specific enhancers along the A-P axis. If this were true, then we hypothesized that we should be able to transform a segment into another, simply by turning on the cell/tissue-type specific enhancers of domain in an area where they are normally off. Using our InSIRT method, we could do this by exchanging initiators from different domains.

For these experiments, we chose to remove the 927 bp *iab-6* initiator and replace it with the molecularly identified initiator from *iab-5*. The *iab-5* initiator is defined as a 1 kb DNA fragment (called IAB5) that, when cloned in front of the *Ubx-lacZ* reporter gene, activates strong β-galactosidase in a pair-rule fashion from PS10.
Therefore, if the domain model is correct, by replacing the \textit{iab-6} initiator with that of \textit{iab-5}, we should be able to activate the enhancers required for PS11/A6 development in PS10/A5. As the difference in the expression pattern of \textit{Abd-B} between PS10 and PS11 can be summarized as PS11 having a higher level of \textit{Abd-B} expression than PS10 (and in more cells), we would expect ectopic activation of \textit{iab-6} to be epistatic to the activity of \textit{iab-5}. In other words, we expected to see a posterior transformation of A5 into A6. As predicted by the domain model, the swapping of the \textit{iab-6} initiator with that of \textit{iab-5} results in a dominant A5 to A6 transformation that is stronger in homozygous flies. This type of posterior-directed abdominal transformation in the BX-C is typically called a Frontal abdominal (Fab) transformation. For this reason, we have named this new mutation, \textit{Fab-6}\textsuperscript{A6IAB5}. Figure 5C shows an abdominal cuticle of a \textit{Fab-6}\textsuperscript{A6IAB5} homozygous male. In this fly, we observe an A6-like lack of A5 sternite bristles, an A6-like trichome pattern on the A5 tergite and a PS11/A6-like \textit{Abd-B} pattern of expression in PS10/A5 (Figure 5F). The fact that A5 appears to be a copy of A6 suggests that everything required for the patterning of A6 is still present in the modified \textit{iab-6} domain, but that these elements have simply been activated one segment too-anterior. These findings strongly support the model in which

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure2}
\caption{InSIRT. A. Step one: Homologous Recombination. The original “ends-out” donor vector (pW25) was modified to contain an \textit{attP} insertion site and a removable \textit{yellow} reporter gene. Using the \textit{yellow} reporter, homologous recombination events could be identified by screening for flies with \textit{yellow} expression in the A5 and A6 segments (a consequence of having \textit{yellow} inserted in the \textit{iab-5} domain). The \textit{yellow} reporter could then be removed to leave only the \textit{attP} site and a single \textit{loxP} recombination site (white triangle) in place of \textit{iab-6}. B. Step two: Reintegration. Plasmids containing a 288 bp \textit{attB} site, a single \textit{loxP} site, a \textit{yellow} reporter and a version of the 19.3 kb fragment were injected into \textit{iab-5,6CI} embryos expressing a maternally supplied \textit{PhC31} integrase [36]. Integration events were isolated based on \textit{yellow} gene expression, then crossed to the Cre recombinase to remove the \textit{yellow} gene and all vector backbone sequence.}
\end{figure}
mutation, A6 seems to be transformed towards A4. Although very similar, we must note that the boundary to an anterior-directed transformation (probably LOF) is incomplete and not seen in the embryo. E. An adult male cuticle of a fly homozygous for the iab-5,6CI chromosome with A5 and A6 transformed towards A4 (notice the A4-like pigmentation on the tergites and the bristled sternites). D. The embryonic nerve cord of homozygous iab-5,6CI embryos shows only a transformation of A6 into A5, as seen by the repetition of PS10/A5-like Abd-B levels in PS11/A6, indicating that the inactivation of iab-5 is incomplete and not seen in the embryo. E. An adult male cuticle from a fly homozygous for the iab-5,6CI rescue chromosome, where the entire 19.3 kb area deleted in iab-5,6CI, looks completely wild type. F. The complete rescue is confirmed by the wild-type pattern of Abd-B in the embryonic ventral nerve cord.

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initiators function, not as enhancers to directly control homeotic gene expression, but rather as domain control centers to turn on the other cis-regulatory elements in a domain.

Interestingly, in the Fab-6cisH5 mutation, A6 seems to be unaffected by the IAB5 swap. As mentioned above, the IAB5 fragment drives reporter gene expression in a pair-rule manner from PS10/A5 (i.e. not in PS11) [12]. In fact, reporter transgenes carrying the entire iab-5 domain are still only capable of driving reporter gene expression in a pair-rule fashion [22]. If this assay reflects IAB5 activity in vivo, then what turns on iab-6 in PS11/A6? Currently, we do not have a completely satisfying answer to this question. However, previous genetic studies tell us that the iab-5 cis-regulatory domain is indeed capable of working in PS11/A6. In iab-6 mutants, for example, A6 is transformed into A5 [22,42]. Thus, it seems clear that our interpretation of IAB5 activity from the transgenic reporter assay oversimplifies IAB5 function. This is perhaps not too surprising, as the reporter gene assays were designed to simply test if a DNA fragment is capable acting as an enhancer. From our experiments, however, it seems that initiators may have a more complex function that is not reflected in the transgene assay.

Mutations affecting Fab-6 boundary function

During our dissection of the iab-6 domain, we also created a number of deletions affecting Fab-6 boundary function. Boundaries function to keep domains autonomous. Based on what was observed in other boundary deletions, we know that boundaries prevent both ectopic activation of posterior domains by elements present in anterior domains (initiators), as well as, prevent anterior domains from being silenced by posterior silencing elements (PREs) [18,31]. The removal of a boundary element, therefore, results in a mixed transformation, where clones of cells in anterior segments become transformed towards cells of more-posterior segments, and clones of cells from posterior segments become transformed towards cells of more-anterior segments. Using our series of deletions, we have narrowed down the Fab-6 boundary to an ~650 bp region of the BX-C.

Previous work from our lab [22] genetically mapped the Fab-6 boundary to an ~4.5 kb region of the BX-C between the distal (relative to the Abd-B transcription unit) breakpoints of the iab-6H1 (3R:12712604) and Fab-6,71 (3R:12708067) mutations. By using deletions nested on the Abd-B-distal side of the iab-5,6CI mutation, we were able to quickly narrow down the location of the Fab-6 boundary further. The first deletion we will speak about, iab-5H1, removes an ~1.2 kb region from the distal side of iab-5,6CI but displays no visible phenotype. This indicates that neither the iab-5 cuticle enhancer (see above), nor the Fab-6 boundary element is removed by this deletion. On the other hand, two bigger deletions, removing ~3.2 kb and ~8 kb respectively (Fab-62 and Fab-61, show mixed anteriorizing (LOF) and posteriorizing (GOF) transformations of A5 and A6 towards A4 or A6. An example of this (Fab-62) can be seen in Figure 6A where we see a loss of bristles on the A5 sternite (indicative of a posterior-directed transformation of A5 to A6), and a loss of pigmentation on the A5 tergite (indicative of an anterior-directed transformation of A5 to A4). Meanwhile, in the A6 segment of each mutant, we see a gain of bristles on the sternite and a loss of pigmentation on the tergite, both indicative of an anterior-directed transformation (probably towards A4). Although very similar, we must note that the Fab-62 mutation displays a slightly stronger GOF phenotype than Fab-61 (data not shown). Consistent with this finding, a PRE has recently been mapped to the region differentiating the Fab-61 and Fab-62 mutations [43]. Thus, if Fab-6 functions like other boundary mutations, the enhanced Fab-6 GOF phenotype is probably caused by deleting this silencing element and shifting the balance between GOF and LOF phenotypes.

The smallest mutation we created that displays an Fab-6 phenotype is Fab-6 in which deletes an ~2 kb span of DNA between the proximal breakpoints of iab-5,6CI (3R:12706385) and Fab-62 (3R:12708661). As expected Fab-62 displays a phenotype similar to Fab-61 and Fab-62 (Figure 6B) with mixed gain- and loss-of-function phenotypes. Meanwhile, a deletion spanning the PRE
region that differs between Fab-61 and Fab-62 shows no phenotype (iab-6D7, Figure 1), suggesting that the critical elements required for Fab-6 function are all contained in Fab-63. Thus, when combining our new data with those of the past, we can now narrow down the Fab-6 boundary to an 650 bp region, spanning from the proximal breakpoint of Fab-6,71 (3R:12708067) to the distal breakpoint of iab-6D7 (3R:12708714). Consistent with this mapping, it has recently been shown that this region contains binding sites for the insulator protein dCTCF [44], and that a fragment containing these dCTCF binding sites blocks enhancer-promoter interactions in an insulator reporter assay [45].

Additional mutations in the iab-6 region

Other deletions created for this study are presented here, solely for the purpose of completeness. These additional deletions are depicted in Figure 1 and their phenotypes are summarized in Table 1. Although we will not discuss these mutants in detail, we would like to point out two key issues surrounding these mutations. First, all deletions removing the initiator fragment show a loss of iab-6 function comparable to that seen in iab-6A7. This finding is in agreement with the domain model and our data, which suggests that the initiator is absolutely required for activating iab-6 function in the cuticle and CNS. Second, we have also isolated a number of mutations that show no noticeable iab-6 phenotype. These mutations, iab-6D7, iab-6D5 and iab-6D6, remove a total of ~9.8 kb of iab-6 sequence without dramatically changing the morphology of the adult cuticle or modifying the Abd-B expression pattern in the embryonic CNS (data not shown).

Figure 4. Phenotypes from initiator mutants. Genotypes are as follows: A.–C. iab-6A7, D.–F. iab-6A5 and G.–I. iab-6A8. A., D. and G. show adult male cuticles. B. E. and H. show pseudo-darkfield views of the fifth and sixth tergites to visualize the trichome patterns. C., F. and I. show the Abd-B staining pattern in the embryonic nerve cord. In wild-type flies, A5/PS10 differs from A6/PS11 based on the sternite shape, the bristles present on the A5 sternite, the trichome pattern on the fifth and sixth tergites, and the Abd-B staining pattern in the CNS (see Figure 3 and Figure 4). The iab-6A5 and iab-6A8 show transformations of A6 to A5 for all phenotypes monitored. Meanwhile iab-6A7 shows only a partial transformation of A6 to A5 as seen by the sternite shape and trichome pattern on A6, which remain A6-like.

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The initiator and a hierarchical structure to gene regulation

For more than twenty years, much of the work on the BX-C has proceeded on the assumption that the BX-C cis-regulatory regions control homeotic gene expression through a multilayered, hierarchical process, summarized in the domain model. Key to this model was the idea that there existed specialized switch elements to control the activity state of the entire domain. Based on transgenic assays, these switch elements were thought to be special early embryonic enhancers, often called initiators. Although through the years, we, and others, have generated many results consistent with this model, we were never able to directly test initiator function in situ, due to experimental difficulties. Thus, a key prediction of the domain model went untested for decades. Here, we have finally provided the data confirming the key role of the initiator in domain activation.

Besides being important for studies on BX-C gene regulation, our findings highlight the possibility of having elements whose sole function may be to control the activity state of other elements. Although we still do not understand how this is accomplished mechanistically, we believe that it is probably through modifying the local chromatin environment around the enhancers. In fact, taking into account the enhancer activity of initiators in transgenic constructs, we are left with an intriguing and testable model for initiator action. As mentioned above, initiators were first isolated as early embryonic enhancers that turned on reporter gene expression in an A-P restricted manner. It has been known for years that the cis-regulatory sequences of the BX-C are transcribed in a parasegment-specific manner where transcripts from each cis-regulatory domain are expressed along the A-P axis in correspondence to where a domain is expected to be active [46–49]. In other experiments it has been shown that forced transcription across PREs in the BX-C can prevent Pc-dependent silencing, and hence, activate a domain [50–52]. Thus, by combining these findings, it is possible to imagine a causal relationship between the initiator and transcription, and the transcription of a domain and domain activation. Accordingly, we propose that initiators might act as enhancers, responding to gap and pair-rule gene products to activate transcription from promoters within the cis-regulatory domains. In doing so, they would indirectly activate homeotic gene expression by preventing the Pc silencing of homeotic gene enhancers. Using the tools developed here, we are now in the process of testing this model.

Another question that we can now address using InSIRT is whether or not initiators are required later in development. Thus far, we have been discussing initiators as only functioning early in development. It is still possible, however, that initiators are constantly required for domain activation or that they play a later role in the regulation of homeotic gene expression. Although the initiator being constantly required to keep a domain active cannot be ruled out, based on our current understanding of initiator function, we do not believe this to be the case. Perhaps the strongest evidence supporting this belief comes from transgenic assays. In transgenic assays, initiator fragments seem to respond to

Figure 5. Phenotypes from initiator mutants. Genotypes are as follows: A. and D. iab-6^4, B. and E. wild type, C. and F. Fab-6^ABS. A–C. Show the ventral sternite cuticles made from adult males, homozygous for the genotype indicated above. Notice that A5 differs from A6 based on the sternite shape and the bristles present on the A5 sternite. The opposite homeotic transformations are highlighted by the direction of the arrows on the left and the right of the cuticles. D–F. Show ventral nerve chords made from homozygous embryos of the genotypes indicated above. Parasegment borders are marked to the left.

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the maternal, gap and pair-rule gene products. Upon disappearance of the early expression pattern of these proteins, initiator activity in transgenes often disappears, or produces a pattern of expression not restricted along the A-P axis (if not paired with a PRE/maintenance element). Based on this, we believe that their role in coordinating the activity of a domain is probably limited to early development. However, this does not mean that all initiators would have no activity outside of initiation phase, only that their function in domain initiation would be limited to early embryogenesis. As mentioned above, the pattern of reporter gene expression driven by some initiator fragments degenerates into a cell-type specific enhancer-like pattern later in development. Therefore, it may be possible that DNA fragments with initiator function may also contain cell-type specific enhancers.

InSIRT as a method to study gene expression

The transgenic reporter assay has played an important role in shaping our understanding of eukaryotic gene expression [53]. Its advantages stem from its speed and the cleanliness of the approach in isolating cis-regulatory elements away from competing or obfuscating signals. To gain these advantages, reporter assays must make a number of critical assumptions. First, they must assume that an activity performed by an element in the reporter assay, is the activity performed by the element in vivo. Second, they must assume that critical transcriptional activities can be tested using the molecular construct devised. And third, they must assume that it is through the addition of these cis-regulatory activities that controlled gene expression is achieved. However, these assumptions are not always correct. Although in the study of the BX-C, transgenes have been extremely useful in estimating the activity of elements, our work on the initiator and our previous work on boundary elements [54] highlight how sometimes the activity seen in transgenes assays only represents a portion of an element’s activity in vivo.

InSIRT is a complementary approach. Relative to the transgenic approach, InSIRT has one main advantage: it tests for changes on biologically relevant targets without necessarily simplifying or assuming an activity. This is key when trying to understand unusual regulatory elements, like initiators. Furthermore, this advantage can be achieved with only a small penalty in time, as, once the homologous recombination has been performed, InSIRT mutagenesis takes only as much time as establishing a single transgenic line. Because transgenic approaches often require the analysis of multiple lines to control for genomic position effects, this penalty is further reduced. Thus, we believe that InSIRT offers scientists a powerful new tool that can be used in combination with classical transgenic methods to better study gene regulation.

Materials and Methods

Fly methods and phenotypes

All crosses, and cuticle preparations were performed using standard Drosophila methods. Abd-B antibody staining was performed as in [22]. Abd-B monoclonal antibody was purchased from the “Developmental Studies Hybridoma Bank” at the University of Iowa. Injection experiments were performed using cleaned DNA preparations (Qiagen) and injected into the iab-5,6CI flies stocks containing an X chromosome expressing the wC31 integrase enzyme under the control of the vasa promoter [36] see [http://www.frontiers-in-genetics.org/flyc31/].

Phenotypes depicted are representative of the genotypes shown. As some of the boundary phenotypes seem to be clonal in nature, there is an occasional variance in the exact number of bristles and the exact pattern of trichomes. We have, therefore, attempted to choose an average representative cuticle for display. Otherwise, the phenotypes can be considered 100% penetrant.

Homologous recombination

Creation of a donor vector for homologous recombination: An AscI-NotI fragment containing the yellow reporter gene flanked by the two loxP sites, and a 255 bp attP integration site was cloned into pW25 digested with AscI-NotI to create the pY25 plasmid. Homology regions of ~4 kb were created by PCR using the following primer pairs: IAB7-AvrII: CCTAGGGCGCGAAGAG-
TAGGGGAAG and Fab7-Asel: CAGC-AAAAATCAGTTAAAAAG, and IAB5-NotI: GCGCAGCGGCTAGTAAAACGGGTGCCC and IAB5-SpiHI: GCATGCACTGGGAGCATTTCATC. These homology regions were then cloned into the pY25 vector in the AscI and SfiI sites or the NotI and SfiI sites respectively. The resulting P-element vector, Py-del, was injected into yw flies and transformants were isolated as yellow flies. Homologous recombination was performed using two independent transformants and the ends-out homologous recombination method of Gong and Golic [34].

Potential homologous recombinants were isolated based on their yellow pigmentation limited to the segments posterior to and including A5 (as a result of being in the iab-5). Genomic southern blots, however showed that all identified events were aborted recombination events in the BX-C. As aborted events happened on each side of the targeted region, we were able to generate the final “planned” deletion by recombining two chromosomes each having recombined properly at one homology region. This recombination was mediated by the Cre recombinase in the loxD sites left behind after removal of the yellow reporter gene. The final chromosome, iab-5,6CI', was verified by genomic southern blot and sequencing.

Generation of integration vectors
A base vector containing the 19.3 kb area deleted in the iab-5,6CI' deletion, a 288 bp attB sequence, a yellow reporter, and one loxP site (called Ky-iab6H) was generated using gap-repair recombineering ([55,56] see Figure 1B). For this, ~500 bp PCR products were generated to both the iab-5 and iab-6 regions (each starting at the breakpoints of the iab-5,6CI' deletion) using the following primers:

iab-5 N: ATAAAGATCCGCGCCCGGTGGTGTTCATCTATAG
iab-5 new (+PmeI): CTCCACCATAGACCCCACTGGTTA-
AGTGTCCGTCGCGAATAGCAACCAG

Table 2. Oligos used to generate the deletions.

| Sequence | Dist |
|----------|------|
| TGCCACTACCCAGACCGACCTACCCAGGGATTTATATGAAAGATGGAATACAAAGCTTTGTCAGAGG | D5 |
| CAGAAGTATGAAATACATTTATATTTCTAATACCAGCAGGATTTCTGGAATACAAAGCTTTGTCAGAGG | D6 |
| TAGCAGCGGACCATCCTGCGCAGAAATCTTAGAAATACCAGCAGGATTTCTGGAATACAAAGCTTTGTCAGAGG | D1 |
| GACTAATTCAAAGCTAGAGGAGGCTGATATTTATTTCTTATGGAATACAAAGCTTTGTCAGAGG | D2 |
| CGGCCAGCAGCTTGGGAGAATTTGCGGATCTGTTAATCTGTGTTAATGGAATACAAAGCTTTGTCAGAGG | D3 |
| AAATGCAGGACTGCGCATGCGAGGCGTGTCTGGTCGCTGGGCTGAGG | D7 |
| CCAGGGTCCACAATGGGCGGACGGGTGCTGACGACGGGCAATCTGACCGATTCAGCAGG | D4 |
| GGGCGCCCCGCGCGCGCGCTTTTGGGGTGGGGAATACAAAGCTTTGTCAGAGG | D8 |
| TGCCGGTATCGACAGATTCTTCTTGGGCTTAGAAGAATTTTCTAGACGGTGCAAGTACTCATCAGG | P2 |
| GTGCACTGGTTTTCTCAAGTAAATGAGATTAATATATATTTACTTGTGCTCCGCGGCGATCTTCTAGAGG | P3 |
| CAAACTGATTTAGAACTGACCGCATCAACACCCCGCGTGCCTGGCGGCGGCTGGTCGCTAGG | P6 |
| GACAGAAGACCACCAACTGACCCGGGCTGAGGAATAGATGCTCATCGACGGCCGCCGGGATCTTCTAGAGG | P4 |
| TGGGCAACAGAAGTTGAGATGCTGGTGGTGGGCGAATATATACAAATGTCGGCTGGGCGGCTTTTCTAGAGG | P1 |
| CTTGGGGCGGCGGTGCACGTTTTCATATTTTGGCAGAAAGTTGCTGGTGGTGGGCGGCTGGGCGGCGGCTTTTCTAGAGG | P5 |
| TGGGGGTTATTAAAGAAAGTTGTTTGCGGCGGCAATTTTACGCGGAAATACAGCTCGGCCGGGCGATCTTCTAGAGG | P7 |

Bold sequences correspond to the FRT-kanamycin-FRT sequences used to prime the amplification of the FRT-kanamycin-FRT cassette. Regular characters correspond to the homology regions used to generate the deletions by recombineering. P1–P7 correspond to the oligos used to generate the proximal breakpoint of the deletions (relative to the Abd-8 promoter), while D1–D8 correspond to the oligos used to generate the distal breakpoint.

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The IAB5 initiator was cloned between these regions of homology using the Ascl restriction site to create the vector, Target iab5(+). An FRT-Kan-FRT cassette was amplified by PCR using the following primers:

- Target iab5(+), to make Target iab5(+), Kan FRT
- sub1-AscI: 5'-AGCTTACATTTTGATAGCTTAAGTGGAT-
- sub2-NotI: 5'-ACTCGCGGCCGCTCGGAAACATCAAAG-
- sub3-AscI: 5'-ACTCGCGGCCGCGAGAATGTTAATATTATTT-TTATAC-TCC3'
- sub4-NotI: 5'-ACTCGCGGCCGCGAGAATATATTATATTCTT-GTGCCAG-GGACC3'

This cassette was then cloned into a unique SnaBI site within the 1.7 kb homology domain (245 bp from the IAB5 initiator) of Target iab5(+), to make Target iab5(+). To recombine the following primers:

- 5'- AGCTTACATTTTGATAGCTTAAGTGGAT
- 3'- AGCTTACATTTTGATAGCTTAAGT

Using the following primers:

- Target iab5(+)
- Kan FRT
- Wrote the paper: FK RKM.

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