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Review

Unraveling *cis*-regulatory mechanisms at the *abdominal-A* and *Abdominal-B* genes in the *Drosophila* bithorax complex

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This review article is dedicated to Edward B. Lewis
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Abstract

Genome sequencing has revealed that in metazoans, only a small percentage of DNA actually codes for functional proteins. Research efforts have focused on elucidating the purpose of the rest of the genome, which was initially largely thought of as mere 'junk' DNA. One genomic region that is proving to be a rich source of new information is the *Drosophila* bithorax complex (BX-C). At this homeotic gene complex, many different classes of *cis*-regulatory elements, such as insulators, silencers, enhancers, and promoters, work together to tightly control gene expression during development. Recent studies have begun to unravel the intricate nature of these regulatory interactions. The BX-C was first discovered and characterized by Ed Lewis over three decades ago. In his seminal 1978 Nature paper, Lewis speculated that "substances" originating from the nongenic regions of the BX-C may regulate expression of the neighboring *abdominal-A* and *Abdominal-B* homeotic genes. A number of discoveries in the last few years suggest that he was right. The activation of some of the *cis*-sequences at the complex appears to be controlled by nongenic transcription, providing a further level of regulatory complexity to regions of nonprotein coding DNA. The hope is that these studies of gene regulation at the BX-C in the humble fruit fly will provide clues as to how vast intergenic regions contribute to the incredible complexity of gene regulation in other species, including humans.

Keywords: *Drosophila*; Bithorax complex; Insulator; Promoter; Enhancer; *cis*-regulation; Nongenic; Transcription

Introduction

Scientific theories are in a constant state of flux and are continuously built up and destroyed as new discoveries are made. The elucidation of the structure of DNA in 1953 (Watson and Crick, 1953) set a foundation upon which scientists have constructed a delicate framework outlining how we believe this DNA is used in development to generate a living, breathing organism. A few years later, Crick proposed what became known as the central dogma of biology, DNA → RNA → Protein, a textbook staple taught in science classrooms everywhere for decades. Since then, information gathered using classical genetics, and now through improving technologies in molecular biology, has revealed that this three-step rule is neither universal nor unidirectional. In particular, discoveries in the last few years have demonstrated that RNA molecules are themselves key players in the regulation of gene expression from DNA (Cook, 2003).

More recently, the sequencing of the human genome (Venter et al., 2001) promised to reveal the key to the complexity of species, originally thought to be reflected in the sheer number of genes. However, most recent estimates have revealed a surprisingly lower number of genes than originally anticipated; the estimate of around 30,000 genes accounts for less than 3% of the total DNA in the human genome (Human Genome Sequencing, 2004). While there has always been some speculation that the regulatory roles for the genome may be critical for development (Davidson, 1999), the rest of the genome was initially largely considered 'junk' DNA, essentially because its purpose had not yet been identified. Results from intense exploration of this genomic frontier over the last few years suggest that these vast regions of nongenic DNA may be

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critically important in the *cis*-regulation of protein-coding genes. In addition, it is now estimated that the noncoding transcription units in the human genome may outnumber the genes by at least tenfold (Kapranov et al., 2002). A number of exciting recent discoveries from studies of the *Drosophila* bithorax complex (BX-C) have provided evidence of nongenic transcription and a complex array of *cis*-regulatory elements that act in concert to control gene expression during development of the embryo.

Cis-regulation of promoter–enhancer interactions at the BX-C

The correct pattern of expression of the homeotic genes is essential for the determination of cell identities along the anterior–posterior axis of the developing embryo in many animals. Discovering how these patterns of gene expression are initiated, maintained, and regulated at the molecular level is critical to our continued understanding of the fundamental processes of developmental biology. The homeotic genes are expressed very early during development and act in a well-defined hierarchical cascade of gene expression. The tight temporal and spatial control of their transcription is critical for normal embryogenesis. The two homeotic gene complexes in *Drosophila* are the Antennapedia complex (ANT-C) and the Bithorax complex (BX-C). The genes located in the ANT-C are responsible for regulating segmental identities in the head and anterior thoracic segments of the fly, while the BX-C genes regulate the patterning of the thorax and abdomen. The BX-C spans approximately 300 kb of genomic DNA but consists of only three homeotic genes: *Ultrabithorax* (*Ubx*), *abdominal-A* (*abd-A*), and *Abdominal-B* (*Abd-B*). Regulatory mutations that alter the expression of these homeotic genes can cause dramatic developmental phenotypes. Perhaps the most famous of these mutants is the 'four winged' fly (Fig. 1A), identified by Ed Lewis (Fig. 1B), in which the pair of halteres located on the third thoracic segment is transformed into a pair of wings. This phenotypic transformation results from a combination of three *cis*-regulatory mutations that alter expression of the *Ubx* gene (Lewis, 1978).

The transcription of the genes in the BX-C is regulated by intervening DNA sequences that can be divided into nine specific domains: *abx/bx*, *bxd/pbx*, *iab-2*, *iab-3*, *iab-4*, *iab-5*, *iab-6*, *iab-7*, *iab-8,9* (Duncan, 1987). Each *iab* domain is thought to contain at least one *cis*-regulatory sequence, known as an enhancer element, which interacts with a single target promoter to drive transcription of one of the neighboring homeotic genes. These enhancers are activated early in development, prior to the establishment of homeotic gene expression, and are thought to be required for the initiation of expression of the homeotic genes in the developing embryo. The expression patterns directed by the iab enhancers can be visualized by looking at the fundamental metric unit for the developing embryo, the parasegment (PS). The identity of each PS is specified by a particular *iab cis*-regulatory domain via activation of the BX-C homeotic genes (Fig. 2). For example, the *cis*-regulatory domains *iab-5* through *iab-8* each regulate the expression of *Abd-B* in PS10-13, respectively (Celniker et al., 1990).

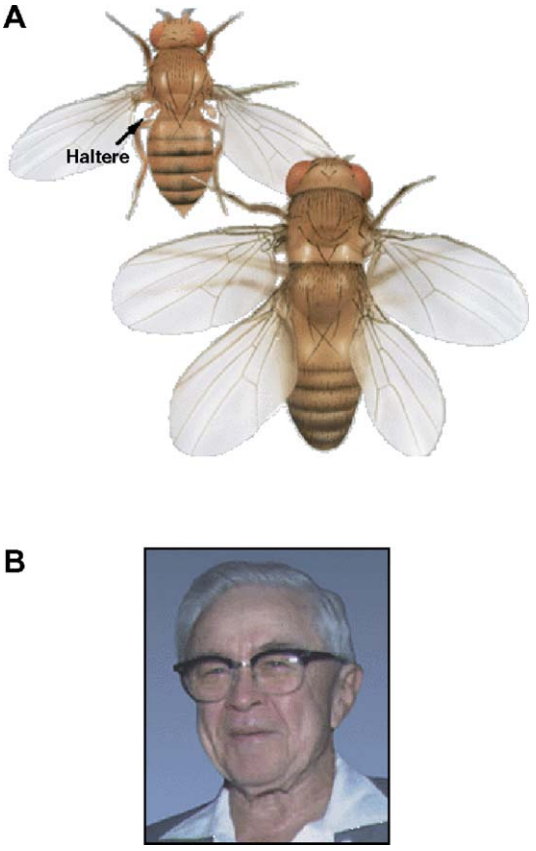

Fig. 1. Bithorax homeotic mutation at the BX-C, identified by Ed Lewis. (A) A normal wild-type *Drosophila* has a pair of wings at its second thoracic segment and a pair of halteres, required for balance in flight, at the third thoracic segment. The four-winged fly results from a combination of three mutations in *cis*-regulatory sequences that control expression from the *Ultrabithorax* gene. These mutations transform the third thoracic segment into an additional second thoracic segment (halteres into wings). (B) E. B. Lewis, shown here at the awarding of his Nobel Prize in 1995, identified the four-winged fly and his pioneering studies on the BX-C were fundamental to our understanding of how these genes control development.

A number of key mechanistic questions relating to the regulatory potential of the iab enhancers remain to be answered. How are the enhancers initially primed for activity in the earliest stages of development? How do the enhancers find their normal target promoter? How are the enhancers restricted to directing expression from a single homeotic promoter throughout development? A set of recent papers have begun to address these critical issues and in the process have revealed some of the intriguing complexity which regulates transcription at the BX-C.

Functional role of insulators

In order for the *cis*-regulatory domains of the BX-C to properly specify segmental identity, they must function autonomously of one another. A special class of *cis*-acting elements known as insulators, or boundary elements, play a

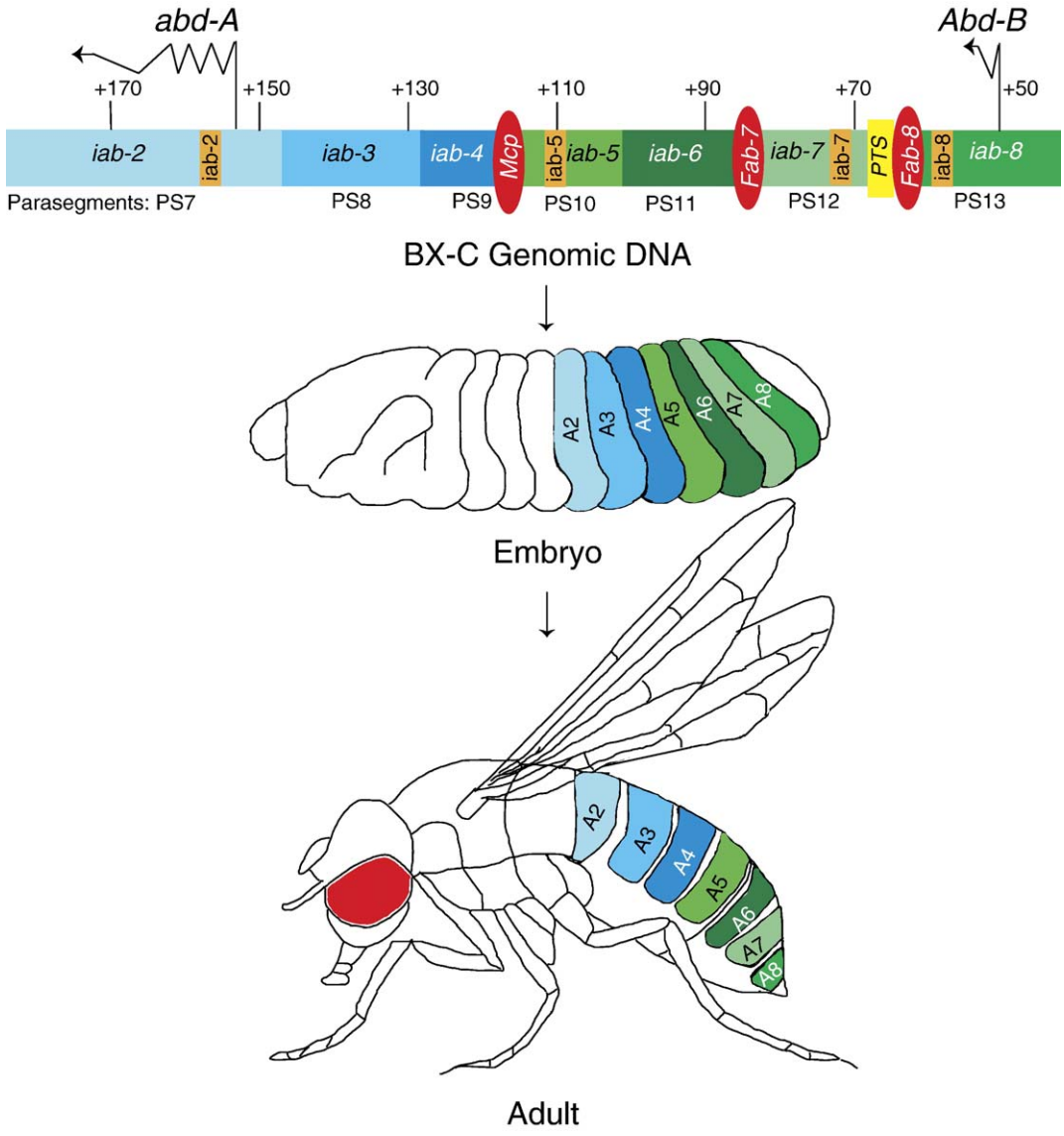

Fig. 2. An extensive array of *cis*-regulatory elements direct embryonic expression of the BX-C homeotic genes. The *abd-A* and *Abd-B* transcription start sites are indicated by leftward arrows. The *iab*-domains (2–8) are indicated as colored rectangles, with each characterized enhancer in the individual *iab* regions specified with an orange rectangle. The *cis*-regulatory interaction between each *iab* domain and their target promoters are specified by color. *Iab-2*, *iab-3*, and *iab-4* regulate *abd-A* (blue), while *iab-5*, *iab-6*, *iab-7*, and *iab-8* interact with *Abd-B* (green). The positions of the Fab-7, Fab-8, and Mcp insulator elements are indicated as red ellipses. The PTS element is indicated by a yellow rectangle. The segmental identities specified by each *iab*-domain are shown in the *Drosophila* embryo and the adult fly. Numbers above line refer to kilobase positions in DNA sequence accession number: DM31961.

critical role in ensuring the genetic autonomy of each iab domain. These elements are functionally capable of preventing enhancer–promoter interactions and buffering transgenes from chromosomal position effects (Kellum and Elgin, 1998). Insulator elements have been identified in several eukaryotic organisms including yeast, *Drosophila*, and vertebrates. The insulator elements identified in *Drosophila* include the *gypsy* insulator isolated from the *gypsy* retrotransposon (Corces and Geyer, 1991; Parkhurst et al., 1988), the scs and scs' insulators flanking the two 87A7 *hsp70* genes (Kellum and Schedl, 1991; Udvardy et al., 1985), SF1 located in the ANT-C (Belozerov et al., 2003) and, from the BX-C, Frontabdominal-7 (Fab-7), Frontabdominal-8 (Fab-8), and Miscadastral pigmentation (Mcp) (Fig. 2).

The insulators at the BX-C play a number of distinct functional roles. The Mcp element from the BX-C is thought to maintain functional autonomy of the *iab-4* and *iab-5* *cis*-regulatory domains. The Mcp element has bipartite function, as it can act as both an insulator and a transcriptional silencer during development (Karch et al., 1994; Mihaly et al., 1998). Recently, Gruzdeva et al. identified the insulating activity in the Mcp element in a minimal 340-bp sequence which maps to the center of the core 755-bp Mcp sequence (Gruzdeva et al., 2005). This minimal element blocks the activating properties of enhancers on the *yellow* and *white* genes when appropriately placed on transgenes. Interestingly, when two copies of the 755-bp Mcp element are placed between the *white* enhancer and *white* promoter, the insulating activity of the Mcp elements is

diminished (Gruzdeva et al., 2005). Similar observations came from previous experiments which demonstrated that when two copies of the *gypsy* insulator element are placed between a promoter and an enhancer, the enhancer-blocking function is abolished (Cai and Shen, 2001; Muravyova et al., 2001). However, the fact that the activities of these insulators are neutralized when paired is not typical of all insulators. For example, Majumder and Cai created heterologous pairs of scs, SF1 and Fab-7 insulators and found that these insulator pairs actually amplified enhancer-blocking function on transgenic constructs (Majumder and Cai, 2003). Their data suggest that different insulators may be capable of working collectively to produce synergistic effects. However, our current understanding of how insulators function at the molecular level is not clear. One hypothesis suggests that insulators may function by sequestering enhancer-binding proteins and therefore inhibiting their activity (Majumder and Cai, 2003). Another idea is that insulators are capable of interacting with each other, causing chromatin rearrangements which may either directly inhibit or even facilitate enhancer–promoter interactions.

It is a long-held belief that insulators in the BX-C are responsible for maintaining the functional autonomy of the *cis*-regulatory information in the *iab* domains. To carry out this function, it is possible that an insulator-pairing mechanism may be operating to strengthen regulatory activities, given that the Mcp insulator element is located in *cis* to the Fab-7 and Fab-8 insulators (Fig. 2). Recent evidence from studies of the proteins responsible for directing the function of the *gypsy* insulator has suggested that inter-insulator communication may in fact be essential for the creation of autonomous chromatin environments (Pai et al., 2004). Perhaps regulated interactions between the insulators in the BX-C are in fact responsible for the generation of specific chromatin loops which, while maintaining regulatory autonomy of the *iab* regions, may also facilitate enhancer–promoter interactions.

Studies examining the Fab-7 insulator have shed further light on the functional regulation of enhancer–promoter communication in the BX-C. Fab-7, the most widely characterized insulator element in the *Drosophila* BX-C, is located between the *iab-6* and *iab-7* *cis*-regulatory domains and functions to ensure the autonomy of these two regulatory regions. A deletion of the Fab-7 element results in a fusion of the *iab-6* and *iab-7* domains (Galloni et al., 1993; Gyurkovics et al., 1990; Mihaly et al., 1997). This fusion event can create two unique outcomes, both of which reflect changes in the autonomy of the *iab* domains. First, positive regulatory elements from the *iab-6* domain can activate the fused *iab-6/iab-7* domain resulting in *Abd-B* being regulated in a PS12 pattern and transforming cell identity from the normal PS11 to PS12. Alternatively, the fused *iab-6/iab-7* *cis*-regulatory domain can be silenced by negative elements from the *iab-7* domain, resulting in the *iab-5* domain regulating *Abd-B* and transforming cellular identity from a PS11 pattern to a PS10 pattern (Hogga et al., 2001). These results indicate that the insulators at the BX-C do more than simply block enhancer–promoter interactions. It would appear that Fab-7 maintains the delicate balance of *cis*-regulatory interactions at the complex. Once this balance is disrupted, the cross-talk between adjacent iab regions can severely influence the regulatory potential of these domains.

Despite their genetic importance, the molecular mechanisms of BX-C insulators have only recently begun to be defined. Structurally, the Fab-7 insulator region contains three major nuclease hypersensitive sites (H21, HS2, and HS3) and also a minor hypersensitive site (Galloni et al., 1993). The functional Fab-7 insulator region extends from the minor hypersensitive site through HS1 and HS2 (Hagstrom et al., 1996; Zhou et al., 1996). The HS1 hypersensitive region contains six GAGA factor binding sites which are arranged in pairs. GAGA factor binding sites are critical to the transcriptional regulation of many *Drosophila* genes including the homeotic genes (Lehmann, 2004). Schweinsberg and Schedl generated mutations in the HS1 region by "hopping" the *bluetail* (*blt*) transposon into the Fab-7 sequence, creating deletions in specific GAGA factor binding sites (Schweinsberg and Schedl, 2004). The Fab-7 mutants they created were still able to initially establish autonomous domains between *iab-6* and *iab-7*; however, they were not capable of sustaining the autonomy as development progressed. Therefore, their results indicated that the Fab-7 insulator contains separable regions that function at different stages during development, and that the combination of these sub-elements is necessary for the normal constitutive activity of Fab-7. The HS1 region is not required for initiation of insulator function but is critical for maintenance of the autonomy of the adjacent *iab* regulatory domains (Schweinsberg and Schedl, 2004). These results are consistent with data showing that functional GAGA factor binding sites are also necessary for the enhancer blocking activity of the Fab-7 insulator on transgenes (Schweinsberg et al., 2004). Future experiments will be required to determine the functional activity of the HS2 region of Fab-7.

The discovery of a number of different insulators in the *Drosophila* genome has raised the issue of whether their activities are identical or functionally distinct. To address this issue, targeted replacement studies have been used to determine the in vivo roles of insulator elements at the BX-C. Hogga et. al revealed that when the Fab-7 insulator element is replaced with either the scs or *gypsy* insulator elements, using gene conversion, the normal functionally autonomous *cis*-regulatory domains are preserved (Hogga et al., 2001). This experiment illustrates that both scs and *gypsy* can insulate regulatory domains in the BX-C in a similar manner to Fab-7. However, unlike Fab-7, both scs and *gypsy* have the additional effect of insulating *Abd-B* from the distal enhancer, iab-5, which normally activates *Abd-B* expression (see Fig. 2). Several models could explain this interaction. Perhaps the inserted insulators constitutively inhibit long distance promoter–enhancer interactions. Another possibility is that Fab-7 itself contains a specific regulatory mechanism that selectively allows enhancers to bypass its insulating activity. There is evidence for such an element at the BX-C, known as the promoter targeting sequence (PTS), which contains an anti-insulator function allowing enhancers to bypass insulators. The PTS is adjacent to the Fab-8 insulator in the endogenous BX-C (see Fig. 2) and is thought to

function by allowing the iab-5, -6, and -7 enhancers to bypass the Fab-8 insulator and activate their target promoter, *Abd-B*. With this in mind, it is possible that the inserted replacement insulators, scs and *gypsy*, are somehow blocking iab-5 and iab-6 interactions with the PTS and consequently inhibiting the ability of these enhancers to bypass Fab-8, thus blocking their directed expression of *Abd-B*.

Anti-insulator elements

The minimal 290-bp PTS is a regulatory element which can facilitate long-range enhancer promoter interactions. It was identified adjacent to the Fab-8 insulator in the BX-C (Lin et al., 2003) (Fig. 2). On transgenic constructs, when placed between an insulator and an enhancer element, this element allows the enhancer to bypass the insulator and activate its target promoter. Also, when more than one promoter is present on a single transgene, the PTS can preferentially limit enhancer activation to a single promoter. However, the mechanism by which this promoter selection occurs is presently unclear (Lin et al., 2003). A possible molecular mechanism for this interaction involves chromatin rearrangement. Chromatin folding into a loop formation may allow the PTS element to create a stable association between the promoter and the enhancer, preventing the enhancer from interacting with other promoters (Lin et al., 2003). If this is the case, then at the endogenous BX-C the PTS could play a role in facilitating the interaction of the distal iab-5 through iab-7 enhancers with their target *Abd-B* promoter (see Fig. 2).

More recently, the PTS has been shown to have even more specialized functions. One experiment suggests that the promoter-targeting function of the PTS has an epigenetic transcriptional memory (Lin et al., 2004). This function was identified by initially integrating PTS-containing transgenes into the *Drosophila* genome and then later relocating them using P-element transposition. The results revealed that enhancers in the transgenes consistently activated the same promoter even when re-inserted into new locations in the genome, suggesting that the PTS has endowed the transgene with a transcriptional memory. The PTS initially requires the presence of an insulator to establish functional promoter–enhancer interactions, but once the transcriptional memory has been set, the PTS can operate in successive generations of cells without the presence of an insulator element (Lin et al., 2004). A third function of the PTS is that it can target more than one enhancer to the same promoter. This indicates that at the endogenous BX-C, the PTS may facilitate the initiation of multiple enhancer–promoter interactions early in development. It is possible that the PTS is acting as a gatekeeper responsible for guiding the iab-5 through 7 enhancers past the Fab-8 insulator to activate their normal target, *Abd-B* (Fig. 2). There is the possibility that other unidentified elements similar to the PTS exist in the BX-C. Perhaps there are elements proximal to the promoters in the BX-C which can tether enhancers to their target promoters. There is evidence of this type of element in the *Drosophila* ANT-C. Calhoun et al. identified a 450-bp tethering element which maps 5′ of the *Sex combs reduced* (*Scr*) promoter that is essential for interactions between *Scr* and the T1 enhancer (Calhoun and Levine, 2003).

There is also evidence of tethering elements working in *trans* to regulate gene expression. A phenomenon known as transvection, first introduced by Ed Lewis in 1954, involves mechanisms that render gene expression sensitive to pairing of homologous chromosomes (Lewis, 1954). During a pairing event, elements such as insulators, silencers, and enhancers have the opportunity to functionally interact with the opposite chromosome. For example, Sipos et al. experimentally illustrated one outcome of transvection at the BX-C by creating deletions 5′ of the *Abd-B* transcriptional start site (Sipos et al., 1998). They reported that these deletions result in the redirection of the *iab-7* regulatory domain to the opposite chromosome in *trans*, with the strength of the redirection correlating to the size of the deletion. In this way, increasingly larger deletions at the *Abd-B* promoter resulted in stronger activation from the iab-7 region on the opposite chromosome. Quite possibly the deletions created upstream of the *Abd-B* gene disrupt tethering elements responsible for maintaining the *cis*-autonomy of the *Abd-B* domain in the BX-C, resulting in the redirection of enhancers like iab-7 to the opposite chromosome in *trans*.

Heritable gene expression patterns

The proper regulation of the BX-C homeotic genes during development is divided into two phases: initiation and maintenance. The initiation phase results in the activation of each *cis*-regulatory domain by interactions between gap and pair-rule gene products and target sequences in each of the *iab cis*-regulatory domains (Muller and Bienz, 1992; Qian et al., 1991). As development proceeds the gap and pair-rule gene products disappear, resulting in a switch in homeotic gene regulation, from initiation to maintenance. The maintenance phase requires the activities of the trithorax-group (Trx-G) and Polycomb-group (Pc-G) proteins, responsible for maintaining active and repressed states of transcription, respectively. Trx-G and Pc-G proteins are recruited to their targets in chromatin by interacting with specific chromosomal elements, called Trithorax and Polycomb response elements (TREs and PREs) (Hagstrom and Schedl, 1997; Paro, 1990; Pirrotta, 1999).

The ability of the Trx-G and Pc-G proteins to regulate intricate patterns of gene expression has led to the idea that these proteins can form multimeric complexes (Papoulas et al., 1998; Petruk et al., 2001; Shao et al., 1999). Once formed, these complexes are thought to be involved in creating heritable epigenetic marks on the chromatin that remain stable through DNA replication and mitosis. These epigenetic marks ensure a stable transcriptional state for genes which are critical to embryonic development, such as those at the homeotic loci in *Drosophila*. More recently, the elements which ensure transcriptional memory by maintaining epigenetic marks have been termed Cellular Memory Modules (CMMs) (Dejardin and Cavalli, 2004). Cellular memory is a conserved mechanism which allows cells to remember the transcriptional state of their established gene expression program throughout development.

The Trx-G and Pc-G proteins are known to interact with DNA regulatory sequences in the BX-C. The Fab-7 HS-3 region contains a 219-bp minimal CMM which regulates expression of the *Abd-B* gene (Dejardin and Cavalli, 2004). In addition, the Mcp element also contains a CMM (Karch et al., 1994; Mihaly et al., 1998). In an exciting recent development, the regulation of these CMMs and the wider *iab* regions has been linked to an extensive nongenic transcription program at the BX-C.

Noncoding intergenic transcription

A number of studies in the field suggest that the *cis*-regulatory elements in the BX-C may in fact be operating in synchrony with a system of intergenic, noncoding RNA transcripts. While it has been known for decades that such transcripts are produced in abundance at the BX-C, their functional role has not been clear. Lipshitz et al. worked with such transcripts as early as 1987, focusing on those produced in the *bithoraxoid* (*bxd*) region of the *Ubx* gene of the BX-C (Lipshitz et al., 1987). Similarly, Sanchez-Herrero and Akam identified substantial transcription through the intergenic region between *abd-A* and *Abd-B* (Sanchez-Herrero and Akam, 1989). Although the generation of these transcripts was observed and their molecular characteristics investigated, no conclusive data could be presented regarding their putative functional role, nor could a comprehensive characterization of the transcription program be performed.

Studies from other genetic loci have indicated that nongenic transcription may be a common feature at tightly regulated gene complexes. Studies at the human β-globin locus revealed a transcription program in which the various regulatory domains are subject to chromatin remodeling via intergenic transcription (Gribnau et al., 2000). More recently, work on the immunoglobulin heavy chain locus in mice revealed a similar active role for noncoding transcription in the alteration of gene function, in which antisense transcription through the V_H region correlates with a switch from DJ_H to VDJ_H recombination (Bolland et al., 2004). The presence of nongenic transcription programs at these distinct gene complexes suggests that the BX-C intergenic transcripts may also have a functional activity. Therefore, addressing the interplay between nongenic transcription and *cis*-regulation could increase our understanding of the regulation of the BX-C.

The creation of a comprehensive profile for the BX-C nongenic transcripts was an important task to direct future research. Knowledge of when and where the transcripts were being generated could provide valuable information as to what role they might be playing in interacting with the *cis*-regulatory elements at the BX-C. In earlier studies, we undertook this task by designing a series of in situ hybridization (ISH) probes spanning from *iab-2* to *iab-8* in the *abdA-AbdB* intergenic region (for an overview of this region, see Fig. 2) (Bae et al., 2002). These studies provided a temporal and spatial map of transcription from this region in the developing embryo and offered some insight into the function of the nongenic BX-C transcripts. Spatially, the transcripts in the embryo are expressed in the same co-linear pattern as their chromosomal organization on the BX-C; that is, the transcripts from *iab-2* are found more anterior to those from *iab-3*, those from *iab-3* found anterior to those from *iab-4*, and so on. Transcription is also contained within individual *iab* chromosomal regions. In this way, one RNA is produced per *iab* region, and the transcription does not appear to traverse the characterized insulator elements. While the expression patterns of all the transcripts have a defined anterior margin in the embryo, the posterior limits can spread into the regions of the other *iab* transcripts. It is interesting to note that almost all of the transcripts are generated from the sense strand, relative to the direction of transcription for *abd-A* and *Abd-B*. If this nongenic transcription was spurious, then the transcripts should be generated randomly from both the sense and antisense strands, showing no strand preference. The predominant transcription of the sense strand and the specific expression patterns in the embryo argue for a functional role for the intergenic transcripts.

Temporal analysis of the transcript patterns reveals further interesting observations. Generally, the transcripts begin to appear in blastoderm (late stage 4 and early stage 5) embryos, prior to the activation of the protein-coding genes of the BX-C (Bae et al., 2002). During this period, the embryo is not yet completely cellularized; however, the RNAs are specifically transcribed in the areas which will correspond to the segments regulated by the *iab* regions in which they are produced. As development continues, the transcript expression patterns shift towards the posterior of the embryo. By stage 9 of development, after the activation of the neighboring homeotic genes, all of the sense transcripts are expressed only in the most posterior two segments of the embryo. The reasons for this persistent collapsed expression pattern are currently unclear.

Taken as a whole, this characterization provides several insights into the functional role of the intergenic transcripts. The fact that they are produced prior to activation of *abd-A* and *Abd-B*, and later collapse into only the last two segments after activation, suggests that the transcripts may perform a preparatory role for the *cis*-regulation of the homeotic genes. Perhaps transcription helps lay down boundaries for the expression of *abd-A* and *Abd-B* and then subsides once the gene expression patterns are established. Alternatively, the nongenic transcription may initiate a regulatory effect which, once established, no longer needs constant maintenance from the early transcripts.

Functional activity for nongenic transcription

While our characterization of the nongenic transcription was ongoing, other groups were investigating the effects of producing ectopic transcripts within the BX-C. In order to explore the function of *cis*-regulatory elements, Bender and Fitzgerald created mutants with P element insertions in the BX-C (Bender and Fitzgerald, 2002). Two separate P elements were used, and both inserted into the distal end of the *bxd* regulatory domain, approximately 10 and 16 kb 3′ of the *abd-A* gene. These mutations had the *Ultraabdominal* (*Uab*) phenotype first discovered by E. B. Lewis (1978), in which the first abdominal segment is transformed into a copy of the second (Bender and

Fitzgerald, 2002). This type of mutation is generally caused by a deletion of a boundary element, which disrupts the autonomy of *cis*-regulatory domains and leads to improper transcription of the BX-C homeotic genes. However, use of ISH probes for regions downstream of the P elements revealed that transcription was occurring from internal promoters in the P elements and spreading towards the *abd-A* gene. This ectopic transcription was passing through the endogenous boundary elements at the *bxd/iab-2* junction in the BX-C and subsequently activating more posterior regulatory domains. The phenotypic effect was therefore not caused by a deletion of a boundary element but rather by transcription through one. As confirmation that this transcription was causing the *Uab* phenotype, the mutant strains were crossed with other P element-carrying strains so as to produce a P cytotype, a condition in which the transcriptional activity of the P elements is repressed. This is believed to be due to the activity of a transposase repressor present in some P elements; as more P elements are introduced, the repressor activity grows, until P element genes are inactivated (Lemaitre and Coen, 1991; Misra and Rio, 1990). Silencing the P-element transcription in this way eliminated the *Uab* phenotype (Bender and Fitzgerald, 2002).

The work of Hogga and Karch, using different experimental procedures, also indicates a similar function for ectopic transcription at the BX-C (Hogga and Karch, 2002). They investigated the functionality of a trimmed-down version of the scs insulator (Hogga and Karch, 2002). A scs fragment 1.2 kb in length was used to replace Fab-7 by gene conversion (Hogga and Karch, 2002). When the new scs fragment, which also contained a promoter, was inserted in an orientation such that the promoter could drive transcription through the PRE adjacent to the Fab-7 region, the proper segmental identity pattern was disrupted in a manner similar to that of a *Fab-7* deletion. The ectopic transcription resulted in a transformation of abdominal segment 6 into segment 7 (Hogga and Karch, 2002). This result signifies that, despite the insulating activity of the scs fragment, the transcription through the adjacent PRE serves to remove its silencing effects, causing the *cis*-regulatory information in the *iab-7* domain to become active anterior to its normal position in the embryo. Our own studies also indicate a functional role for the endogenous intergenic transcription at the BX-C. A deletion at the Mcp region (Lewis, 1978) results in a loss of the nongenic transcription in the adjacent *iab-4* domain, presumably due to inactivation of the promoter for this transcript. The absence of the *iab-4* transcript is correlated with a transformation of abdominal segment 4 into 5 (Drewell et al., 2002). Taken together, these studies suggest that controlled nongenic transcription in the *iab* regions is critical to the proper function of the BX-C and appears to play a role in activating *cis*-regulatory domains during development.

Chromosomal memory

Rank, Prestel, and Paro used a third experimental approach, designed specifically to test the memory function of a PRE. By utilizing a GAL4-inducible promoter linked to the *cis*-elements Fab-7, bxd, or Mcp, they were able to examine how nongenic transcription switches these sequences from an inactive to active state on transgenes and if these states can be inherited in future generations of cells (Rank et al., 2002). In this way, they were addressing whether the *cis*-elements are able to inherit a chromosomal memory, based on their inherited chromatin configuration, during cell divisions in the embryo. Several interesting points emerge from their studies. The first is that all of these elements operate in the previously outlined manner: transcription through the element switches it into an active configuration. However, this switch is only permanent when executed during embryonic stages; activation by GAL4 exposure in larval stages could transiently induce transcription, but when the stimulus was removed, production of the transcripts ceased and the PRE reverted back to its initial silenced state. Another critical point was discovered while working to isolate the core elements of Fab-7 for use in these experiments. When the core Fab-7 insulator and associated PRE element, or the PRE element alone, were inserted on transgenes, they were not transcribed and remained in an inactive state. This argues for the necessity of the entire Fab-7 region in order to generate the transcripts associated with functional switching. These experiments supported the idea that so-called PREs were more than simply responding to Pc-G proteins and therefore could be termed cellular memory modules (CMMs).

More recently, Schmitt, Prestel, and Paro used a transgenic construct containing a Fab-7 PRE to carry out further characterization of the memory function of this element (Schmitt et al., 2005). The data they present support the previously outlined model with some interesting additions. When transcription occurs constitutively through the Fab-7 PRE element, the adjacent *mini-white* gene of the transgenic construct is expressed; when the transcription through Fab-7 PRE is halted, the *mini-white* gene is silenced, even though it has its own promoter. This suggests that the Fab-7 PRE region contains some silencing activity that acts over neighboring regions in the transgene, an activity removed by transcription. They also established that nongenic transcription was necessary throughout embryonic development in order to lock the Fab-7 PRE into an activated state. Thus, when Fab-7 PRE transcription was generated using the *hunchback* promoter, which is active only in early embryonic development, it was not sufficient to lock the region into its transcriptionally active state at later stages of development (Schmitt et al., 2005). Therefore, a longer transcriptional period is necessary to properly switch the PRE to its activating state.

It is known that chromatin can be modified during the course of transcription via acetylation, methylation, and so forth, and that these modifications can act as genetic switches and tags during development (Margueron et al., 2005). The work reviewed here presents evidence supporting the idea that at the BX-C, such chromatin modifications may be connected with nongenic transcriptional activity. This relationship between chromatin modification and nongenic transcriptional activity is not a new one. A similar activity has been suggested at the human β-globin locus (Gribnau et al., 2000). In these transgenic experiments, RNA fluorescence in situ hybridization was used to find distinct regions of nongenic transcription surrounding

the genes of the β-globin locus. As each gene was sequentially activated, there was a shift in the nongenic transcription program. Accompanying this nongenic transcription was a concurrent alteration of local chromatin formation to a more open state, as confirmed by sensitivity to DNaseI. Removal of the nongenic transcription also prevents the opening of the chromatin, leading to disruption of the proper program of switching between transcription of the various globin genes at the locus. This parallels the findings of the studies at the BX-C covered in this review, in which disruption of nongenic transcription has definite effects on the proper developmental sequence of gene regulatory events.

The coordination of nongenic transcription in a developmental program is essential for the proper formation of the developing *Drosophila* embryo; a number of the studies examined here show the effects of ectopic transcription through *cis*-sequences, which act to regulate associated genes. As a general rule the nongenic transcription appears to function in the activation of these regulatory sequences. For example, as transcription proceeds through a CMM from the BX-C, it is switched from the recruitment of Pc-G to Trx-G proteins (Schmitt et al., 2005). This presumably leads to a heritable remodeling of the chromatin environment from an inaccessible, silenced configuration to an accessible and active configuration (Fig. 3). This switch in chromatin structure results in the activation of expression from neighboring genes (Fig. 3).

Grand unifying theory of *cis*-regulation at BX-C?

The critical functional role of the homeotic genes from the BX-C during *Drosophila* development is reflected in the complexity of their regulation. Misexpression of these genes can radically affect the identity of developing segments and, in the most severe cases, create homeotic transformations (see Fig. 1). An intricate network of *cis*-regulatory controls appear to be essential to successfully direct normal homeotic gene expression patterns during development. In particular, the interplay between nongenic transcription and an array of *cis*-elements at the complex seems to be of crucial importance.

The spatial and temporal pattern of intergenic transcription in the *cis*-regulatory regions at the endogenous BX-C suggests a potential role early in embryonic development (Drewell et al., 2002). It is possible that the transcription program serves an activating function similar to that produced by the ectopic RNAs studied by others (Bender and Fitzgerald, 2002; Hogga and Karch, 2002; Rank et al., 2002). The endogenous transcription may prime the *iab* domains early in development, subsequently allowing the existing *cis*-regulatory elements to initiate control of the correct expression patterns of *abd-A* and *Abd-B* in the embryo.

The molecular mechanisms which underpin these functional activities remain to be fully characterized. However, the current evidence points to a potential link with chromatin structural modifications. It is possible that early in *Drosophila* development, the *cis*-regulatory regions of the BX-C are in an inaccessible chromatin structure that prevents recruitment of the *trans* factors required to activate control of homeotic gene expression (see Fig. 4A). The initiation of the nongenic transcription program may then be needed to ablate this repressive state and create a memory-free, 'amnesic' chromosome. How the nongenic transcription is activated so early in development is currently not known. It is possible that the promoters for these transcripts and their own regulatory sequences may somehow be protected from the repressive chromatin environment at the BX-C. However, once the intergenic transcription is initiated, the passage of an RNA polymerase-containing complex through the different *iab* regions may permit the recruitment of *trans*-factors to previously inaccessible *cis*-regulatory elements (Fig. 4B). The RNA polymerase II complex is known to include a histone acetyltransferase (Wittschieben et al., 1999) that could modify the histone tails in nucleosomes across the transcribed regions. It is also conceivable that the RNA polymerase II complex recruits other chromatin-modifying

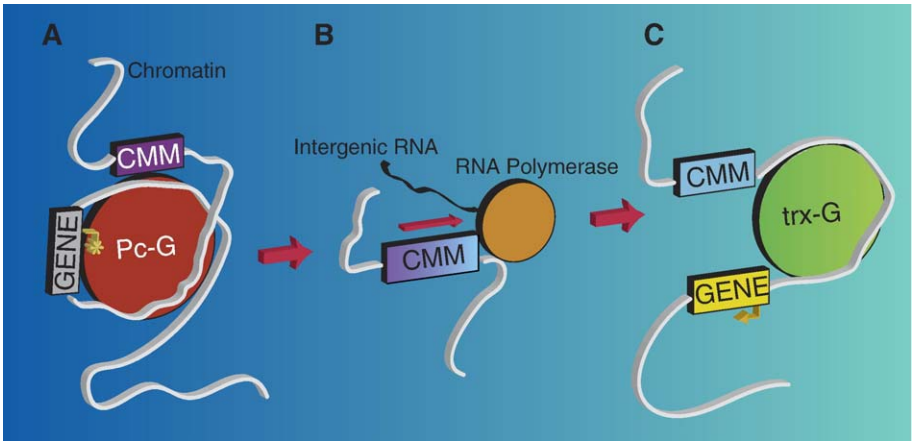

Fig. 3. Nongenic transcription acts as a molecular switch for the Pc-G/trx-G system. Current data indicate that ectopic transcription through Cellular Memory Modules (CMM) in the BX-C switches them from recruiting Polycomb Group (Pc-G) proteins and associated homeotic gene silencing, to trithorax group (trx-G) protein recruitment, with associated activation of the neighboring homeotic genes. (A) The silencing state of a CMM recruits Pc-G proteins and remodels chromatin structure so as to repress the neighboring gene. (B) Nongenic transcription through the silencing CMM causes a switch to an active state. (C) CMM in active state recruits trx-G proteins and remodels chromatin, leading to a gene accessible for transcription.

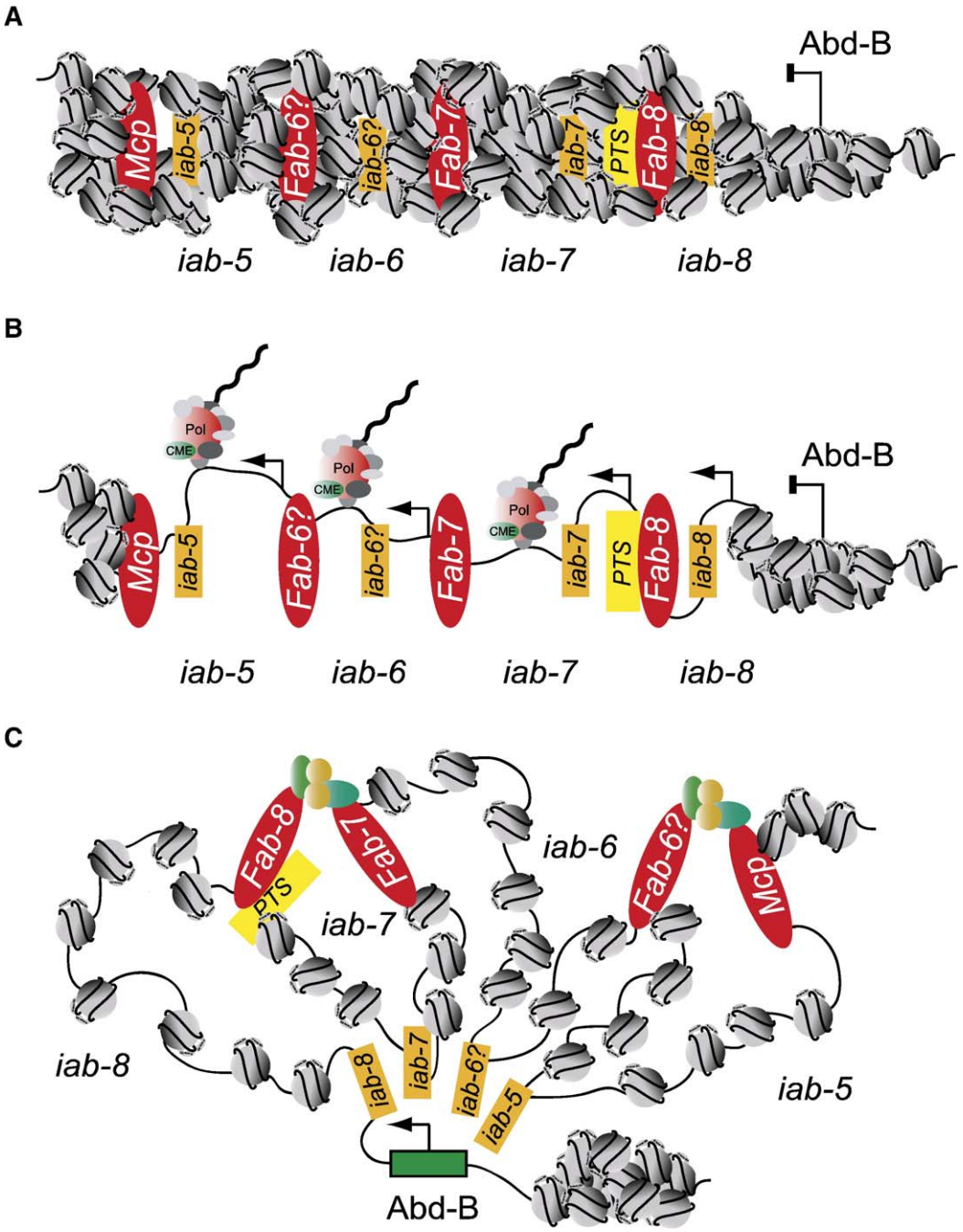

Fig. 4. Model of functional interaction between nongenic transcription and *cis*-regulatory elements for the *Abd-B* gene. (A) In the earliest stages of development, the *cis*-regulatory domains of the BX-C are sequestered in an inactive chromatinized environment. (B) Prior to the expression of the homeotic genes, nongenic transcription at the individual *iab* regions is required for the transition to 'open' chromatin. After the passage of an RNA polymerase (Pol) and associated chromatin modifying enzymes (CMEs), the array of *cis*-elements are able to recruit the necessary *trans* factors to become functionally active. (C) Insulator elements regulate promoter–enhancer interactions by pairing (via unknown proteins) to form chromatin loops which facilitate the recruitment of the iab enhancers to the *Abd-B* gene.

enzymes, such as histone methyltransferases or deacetylases, capable of contributing to the remodeling process. In this way, transcription would facilitate the 'opening' of chromatin in the *iab* regions, correlating with the timing of activation for *cis*-regulatory elements in specific domains of the embryo (Fig. 4B). Once initiation of the functional activities of the *cis*-regulatory sequences has begun, then these elements are responsible for controlling expression from the neighboring homeotic genes. A critical level of control is the regulation of promoter–enhancer interactions. The pairing characterized for a number of *Drosophila* insulators (Majumder and Cai, 2003) may indicate that similar mechanisms are at play for the multiple insulators at the BX-C. Such insulator–insulator interactions are thought to generate specific chromatin loops (Byrd and Corces, 2003; Pai et al., 2004). At the BX-C, such loops could direct and restrict the intervening *iab* enhancer sequences to their target promoter (Fig. 4C), resulting in the required high fidelity of

segment-specific homeotic gene expression in the developing embryo.

Studies from the last few years have generated a rich array of new information that will help us to understand how *cis*-regulation in the *Drosophila* BX-C is controlled; however, it is clear that we are presently far from being able to create a complete picture. Future experiments will undoubtedly continue to reveal the true mechanistic interactions between the nongenic transcription program and the *cis*-regulatory elements at the BX-C. For now, there are still plenty of uncharted frontiers to be explored in the BX-C and across the rest of the recently sequenced metazoan genomes. The prospect that continued study of the BX-C will provide answers to the central questions of gene regulation that can be applied to other complex genetic loci is very appealing. What is certain is that the current theories we believe to be true will continue to be shaped and changed by new discoveries.

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