RESEARCH COMMUNICATION

Stalled Hox promoters as chromosomal boundaries

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Many developmental control genes contain stalled RNA Polymerase II (Pol II) in the early *Drosophila* embryo, including four of the eight Hox genes. Here, we present evidence that the stalled Hox promoters possess an intrinsic insulator activity. The enhancer-blocking activities of these promoters are dependent on general transcription factors that inhibit Pol II elongation, including components of the DSIF and NELF complexes. The activities of conventional insulators are also impaired in embryos containing reduced levels of DSIF and NELF. Thus, promoter-proximal stalling factors might help promote insulator-promoter interactions. We propose that stalled promoters help organize gene complexes within chromosomal loop domains.

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Hox genes are responsible for the anterior-posterior patterning of most metazoan embryos (Lewis 1998). They are typically organized in gene complexes containing a series of *cis*-regulatory DNAs, including enhancers, silencers, and insulator DNAs (Kaufman et al. 1990; Maeda and Karch 2007). In *Drosophila*, the eight Hox genes are contained within two gene complexes: the Antennapedia complex (ANT-C), which controls the patterning of anterior regions, and the Bithorax complex (BX-C), which controls posterior regions (Sanchez-Herrero et al. 1985; Kaufman et al. 1990; Lewis 1998). The proper spatiotemporal transcription of Hox genes is achieved by the coordinated action of linked *cis*-regulatory DNAs that are organized in a colinear fashion across the ANT-C and BX-C complexes.

Chromosomal boundary elements, or insulators, are essential for the orderly regulation of Hox gene expression. They are thought to ensure proper *cis*-regulatory "trafficking," whereby the correct enhancers interact with the appropriate target promoters (Sipos and Gyurkovics 2005; Maeda and Karch 2007; Bushey et al. 2008). Insulators might also help control the levels of transcription by

attenuating enhancer-promoter interactions (Zhou et al. 1996). Insulators are sometimes associated with promoter targeting sequences (PTS) (Zhou and Levine 1999; Akbari et al. 2007; Lin et al. 2007), which can facilitate enhancer-promoter interactions by modulating the activities of neighboring insulators.

Recently, long-range *cis*-regulatory interactions have been mapped in *Drosophila* Hox complexes using the DamID technique (Cleard et al. 2006), chromosomal conformation capture (3C) assays (Lanzuolo et al. 2007), and transgenic approaches (Kyrchanova et al. 2008). These studies suggest that the *Fab7* and *Fab8* insulators are associated with the *Abd-B* promoter under repressed conditions, even though they map >30–50 kb downstream from the promoter (Cleard et al. 2006; Lanzuolo et al. 2007; Kyrchanova et al. 2008). These long-range interactions depend on the CTCF boundary-binding protein (Kyrchanova et al. 2008), thereby raising the possibility that insulators interact with one another and organize *Abd-B* *cis*-regulatory DNAs within chromosomal loop domains. Similarly, the prototypic insulators flanking the heat-shock puff locus, *scs* and *scs'*, have also been shown to interact with one another (Blanton et al. 2003). Additional insulator-insulator loops have also been documented (Kravchenko et al. 2005; Kyrchanova et al. 2007). These loops are thought to facilitate the interactions of remote enhancers and silencers with appropriate target promoters. Here, we present evidence that Hox promoters with stalled RNA Polymerase II (Pol II) possess an intrinsic insulator activity, which might help foster the formation of insulator-promoter chromosomal loop domains.

Results and Discussion

Stalled Hox promoters contain insulator activities

Four of the eight Hox genes contained in the ANT-C and BX-C contain stalled Pol II. Interestingly, all four stalled genes map at the boundaries of the two Hox complexes (see Supplemental Fig. 1; Muse et al. 2007; Zeitlinger et al. 2007). In contrast, internal Hox genes (*pb*, *Dfd*, and *Scr* within the ANT-C, and *abd-A* within the BX-C) lack stalled Pol II. This arrangement of stalled Hox genes raises the possibility that stalling contributes to the chromosomal organization of Hox complexes. All four stalled Hox genes (*lab*, *Antp*, *Ubx*, and *Abd-B*) were tested for enhancer-blocking activity in transgenic embryos, along with the promoter regions of two nonstalled genes (*Scr* and *abd-A*) (Fig. 1). Test promoters were placed 5' of *lacZ* and inserted between a divergent *white* reporter gene and 3' *iab-5* enhancer (IAB5) (see diagrams in Fig. 1).

IAB5 regulates *Abd-B* expression in posterior regions of the early embryo, corresponding to the primordia for parasegments 10–14 (Busturia and Bienz 1993). IAB5 is a robust enhancer, and can activate *lacZ* and *white* even when positioned far from the reporter genes. This assay was used to reveal an intrinsic enhancer-blocking activity of the *eve* promoter region (Ohtsuki and Levine 1998). *eve/lacZ* fusion genes block the ability of IAB5 to activate a distal CAT reporter gene. However, mutagenized *eve* promoter sequences lacking a critical proximal GAGA element failed to block IAB5-*white* interactions (Ohtsuki and Levine 1998). Similarly, the *Abd-B* proximal promoter

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Figure 1. Paused/stalled Hox gene promoters display insulator activity. The Hox gene promoters were tested for their ability to block enhancer promoter interactions in transgenic assays. The Hox/*lacZ* fusions were cloned between IAB5 enhancer and *white* reporter gene. The IAB5-*lacZ* interaction was seen for all of the transgenic lines for *Abd-B/lacZ* (A), *Ubx/lacZ* (C), *abd-A/lacZ* (E), *Antp/lacZ* (G), *lab/lacZ* (I), and *Scr/lacZ* (K) as the classical PS10-14 staining was observed during *lacZ* probe in situ. The IAB5-*white* interaction was blocked in all stalled promoter-*lacZ* fusions like *Abd-B/lacZ* (B), *Ubx/lacZ* (D), *Antp/lacZ* (H), and *lab/lacZ* (J) lines as no *white* in situ signal was observed in the IAB5 pattern. The nonstalled promoter-*lacZ* fusions like *abd-A/lacZ* (F) and *Scr/lacZ* (L), however, did show the IAB5 pattern when stained for *white* probe, as they did not show any insulator activity. The weak staining in the head regions is due to the P-transformation vector used in these experiments (Small et al. 1992). The embryos are aligned anterior to the left and posterior to the right.

(*Abd-Bm*) and *Ubx* promoter regions block activation of distal *white* expression (Fig. 1A–D), whereas the *abd-A* promoter does not interfere with the activation of *white* expression in the presumptive abdomen by the IAB5 enhancer (Fig. 1E,F).

These results suggest that the stalled *Abd-B* proximal promoter and *Ubx* promoters possess an enhancer-blocking activity, whereas *abd-A* does not. A similar trend was observed for Hox promoter sequences from the ANT-C. The *Antp* and *lab* promoters block IAB5-*white* interactions (Fig. 1G–J), whereas the *Scr* promoter (which lacks stalled Pol II) does not interfere with the activation of *white* expression in the presumptive abdomen (Fig. 1K,L). We also examined stalled genes from the *tinman* complex (Tin-C), which encode NK homeobox proteins responsible for patterning mesodermal lineages. All of the stalled promoters from the Tin-C contain insulator activities (Supplemental Fig. 2). In contrast, nonstalled promoters from *lbl* and *C15* lack such activities when tested in similar transgenic assays (J. Cande, unpubl.). Even the *Hsp70* promoter, the classic example of Pol II pausing, displayed insulator activity when tested in similar enhancer-blocking transgenic assays (Supplemental Fig. 3).

Stalled Hox promoters do not display tethering activities

The preceding experiments suggest that stalled Hox gene promoters contain enhancer-blocking activities. However, an alternative possibility is that stalled promoters are “stronger” than the *white* promoter, and are able to sequester the shared IAB5 enhancer. To distinguish be-

tween competition and insulator activities, the IAB5 enhancer was placed between the divergently transcribed *white* and *lacZ* reporter genes (Fig. 2). When the *white* promoter sequence was placed 5' of the *lacZ* reporter gene, the shared IAB5 enhancer worked equally well to activate both *white* and *lacZ* expression (Fig. 2A,B). Similar results were obtained when the leftward *lacZ* reporter gene was placed under the control of either the stalled *Abd-B* (Fig. 2C,D) or *Ubx* (Fig. 2E,F) promoters. In all of these cases, both *white* and *lacZ* are expressed equally well in the presumptive abdomen. These results suggest that stalled promoters do not block enhancer-promoter interactions by a competition mechanism. Rather, they work like insulators and block such interactions only when positioned between the distal enhancer and target promoter.

Hox promoter-blocking activity requires negative Pol II elongation factors

To determine whether stalled Pol II is important for the enhancer-blocking activities of *Ubx* and *Abd-B*, we examined mutant embryos with reduced levels of critical Pol II elongation factors (Fig. 3). *Ubx* and *Abd-B* were selected for further studies since we showed recently that optimal expression of both genes depends on the Pol II elongation factors Cdk9 (pTEFb) and Elo-A (Chopra et al. 2009). We reasoned that destabilization of stalled Pol II might reduce the enhancer-blocking activities of the *Ubx* and *Abd-B* promoter regions. However, reductions in Cdk9 and Elo-A are expected to stabilize, not destabilize, Pol II stalling since both are positive factors that promote elongation (Saunders et al. 2006). Indeed, reductions in Cdk9 or Elo-A activity do not alter the enhancer-blocking activities of the *Ubx* and *Abd-B* promoters (Supplemental Fig. 4).

To investigate the link between Pol II stalling and enhancer blocking, we examined two negative elongation factors: NELF and DSIF (Wada et al. 1998; Yamaguchi et al. 1998; Kaplan et al. 2000; Lee et al. 2008). The NELF-E protein binds to the short nascent transcripts protruding from the active site of Pol II after transcription initiation and promoter clearance, and thereby inhibits Pol II elongation (Wu et al. 2005; Lee et al. 2008). Both NELF and DSIF are thought to help stabilize Pol II at the pause site,

Figure 2. *Abd-B* and *Ubx* promoter insulator activity does not involve tethering. The *Abd-B* and *Ubx* stalled promoters were tested for their ability to display promoter competition in a transgenic assay in which the IAB5 enhancer was cloned in between the *white* reporter and *lacZ* fusion. As a control, the *white/lacZ* fusion (A) and *white* (B) were driven equally well by IAB5 enhancer in a *white/lacZ* (A,B) transgene. The IAB5-*lacZ* interaction (C,E) as well as the IAB5-*white* (D,F) interaction were facilitated in *Abd-B/lacZ* (C,D) and *Ubx/lacZ* (E,F) lines, respectively.

Figure 3. Negative elongation factors are required for Hox gene promoter insulator activity. The stalled promoter insulator lines were tested in elongation factor mutant backgrounds. (A–H) The *Abd-B/lacZ* line was tested in *Nelf-E/+* (A,B), *Nelf-A/+* (C,D), *Spt4/+* (E,F), and *Spt5/+* (G,H) backgrounds, and there was loss of insulator activity of the *Abd-B* promoter in these mutant backgrounds as seen by the appearance of the IAB5 pattern in situ signal when *white* probe was used (B,D,F,H). (I–P) Similar disruption of the insulator activity was observed for the *Ubx/lacZ* transgene in different mutant backgrounds of *Nelf-E/+* (I,J), *Nelf-A/+* (K,L), *Spt4/+* (M,N), and *Spt5/+* (O,P). (J,L,N,P) The *white* probe displayed the IAB5 pattern staining in these elongation mutant backgrounds.

typically 20–50 base pairs (bp) downstream from the transcription start site (Saunders et al. 2006; Gilchrist et al. 2008; Lee et al. 2008). Since Pol II elongation factors are encoded by essential genes, it is not possible to examine the *lacZ/white* reporter genes in homozygous mutant embryos. Instead, the transgenes were expressed in embryos derived from heterozygous females, and thereby contain half the normal levels of NELF and DSIF (Spt) subunits (Fig. 3). Reductions in *Nelf-E* (Fig. 3A,B,I,J), *Nelf-A* (Fig. 3C,D,K,L), *Spt4* (Fig. 3E,F,M,N), and *Spt5* (Fig. 3G,H,O,P) cause clear disruptions in the enhancer-blocking activities of both the *Ubx* and *Abd-B* promoters, as seen by the strong activation of the distal *white* reporter gene (Fig. 3B,D,F,H,I,L,N,P). In contrast, *white* expression is blocked when the same transgenes are expressed in a wild-type background (see Fig. 1B,D). The simplest interpretation of these results is that reduced levels of the NELF and DSIF inhibitory complexes destabilize stalled Pol II at the pause site. Reduced pausing results in diminished enhancer-blocking activities. There is a similar loss in the enhancer-blocking activities of the *eve* promoter (Ohtsuki and Levine 1998) and *Fab7* insulator (Schweinsberg et al. 2004) when the transgenes are expressed in embryos containing reduced levels of the GAGA factor, *Trl* (Farkas et al. 1994). It is conceivable that the GAGA factor also contributes to the enhancer-blocking activity of the *Ubx* promoter since *Trl/+* embryos display augmented expression of *white* (data not shown).

In principle, the augmented expression of the *white* reporter gene might not result from the impaired function of the stalled insulators, but might arise from enhanced activity of the *white* promoter. To investigate this issue, we performed Pol II chromatin immunoprecipitation (ChIP) assays, coupled with quantitative PCR (qPCR) assays (Supplemental Fig. 5). In DSIF and NELF mutant embryos, there is no increase in Pol II levels at either the *white* promoter or intronic regions as compared with wild-type embryos (Supplemental Fig. 5). These results suggest that augmented expression of *white* is due to diminished insulator activities of stalled promoters in

embryos containing reduced levels of negative Pol II elongation factors.

Negative elongation factors are essential for *Fab7* and *Fab8* function

It has been suggested that insulators might work, at least in part, via promoter mimicry (Geyer 1997). To explore this issue, we examined the impact of reductions in NELF and DSIF on the activities of two known insulators—*Fab7* (Gyurkovics et al. 1990) and *Fab8* (Barges et al. 2000)—from the BX-C. Previously published transgenic lines were used that contain *Fab7* or *Fab8* inserted between the IAB5 and 2XPE (*twist*) enhancers (Zhou et al. 1996) attached to a leftward *lacZ* reporter gene and rightward *white* reporter (see the diagrams in Fig. 4). In wild-type embryos, the reporter genes are activated only by the proximal enhancer. Thus, *white* is activated solely in the mesoderm by the 2XPE enhancer (Fig. 4B,L), while *lacZ* is activated in the presumptive abdomen by IAB5 (Fig. 4A,K). The distal enhancers are blocked by the *Fab7* or *Fab8* insulators. Consequently, IAB5 fails to activate *white* and the 2XPE enhancer fails to activate *lacZ*.

Very different results are observed when the transgenes are crossed into mutant embryos containing reduced levels of NELF or DSIF (Spt) subunits. There is a loss in the enhancer-blocking activities of the *Fab7* and *Fab8* insulators and, as a result, *white* and *lacZ* display composite patterns of expression in the mesoderm and abdomen since they are now activated by both enhancers (Fig. 4C–J,M–T). These results suggest that negative Pol II elongation factors are required for the enhancer-blocking activities of the *Fab7* and *Fab8* insulators.

We propose that insulators interact with stalled promoters to form higher-order chromatin loop domains (Fig. 5), similar to those created by insulator–insulator

Figure 4. Negative elongation factors are required for enhancer-blocking activity of putative insulators like *Fab7* and *Fab8*. Enhancer-blocking lines containing *Fab7* (A–J) and *Fab8* (K–T) insulators, cloned between divergently placed IAB5/*lacZ* and 2XPE/*white* reporters, were tested in elongation factor mutant backgrounds. The *Fab7* (A,B) and *Fab8* (K,L) line shows *lacZ* staining in IAB5 pattern (A,K) and *white* in *twist* (2XPE) pattern (arrow) (B,L), respectively. In *Nelf-E/+* (C,M), *Nelf-A/+* (E,O), *Spt4/+* (G,Q), and *Spt5/+* (I,S) backgrounds the *lacZ* in situ now displays both the IAB5 as well as the 2XPE patterns for both *Fab7* and *Fab8* lines, respectively. A similar dual IAB5 and 2XPE pattern was observed for the *white* in situ in *Nelf-E/+* (D,N), *Nelf-A/+* (F,P), *Spt4/+* (H,R), and *Spt5/+* (J,T) backgrounds for *Fab7* and *Fab8* lines, respectively. The embryos are aligned anterior to the left, posterior to the right, dorsal up, and ventral down.

Figure 5. Stalled Hox promoters may help promote higher-order chromatin organization within the Hox loci. Our results suggest that the stalled promoters contain intrinsic insulator activity that requires NELF (N) and DSIF (D) proteins, and this may help define higher-order loops within gene complexes such as the Hox complex. The stalled Pol II (P) along with the NELF and DSIF complex may interact with putative insulator (In) sequences, as seen for the *Abd-B* promoter (stalled) and the *Fab7* (insulator). Our experiments also suggest that that putative insulator sequences also require NELF and DSIF proteins, and this could be due to sharing of these proteins via the formation of higher-order loops. Such loop domains may help in proper regulation of genes and prevent any aberrant activation from neighboring enhancers (En) (shown by dashed arrow), thus favoring proper gene regulations at the higher-order level.

interactions (Blanton et al. 2003; Vazquez et al. 2006; Kyrchanova et al. 2007; Lanzuolo et al. 2007; Bartkuhn et al. 2009). Perhaps proteins that bind insulators interact with components of the Pol II complex at stalled genes. Indeed, the recent documentation that the BEAF insulator protein binds to many of the same sites as NELF is consistent with a physical link between stalled Pol II and insulators (Jiang et al. 2009). The resulting chromatin loops can prevent the inappropriate activation of stalled genes by enhancers associated with neighboring loci (Fig. 5). As discussed earlier, stalled Hox genes are located at the boundaries of the ANT-C and BX-C (Supplemental Fig. 1). This arrangement might help ensure that *cis*-regulatory sequences located outside the complexes do not fortuitously interact with genes contained inside the complex and vice versa. The demonstration that stalled Hox promoters possess an intrinsic insulator activity adds to the intricacy of the chromosomal landscapes (Spitz et al. 2003) that control Hox gene expression in both arthropods and vertebrates.

Materials and methods

Plasmid construction and molecular analysis

The *iab-5* enhancer was amplified as a 1-kb fragment from the genome and cloned into the enhancer-blocker vector. The enhancer-blocker vector was engineered to retain *white* and *lacZ* as markers and the backbone was similar to as described earlier (Ohtsuki and Levine 1998). The paused promoters were selected based on Pol II-binding signals from the ChIP-chip study and usually flanked 100 bp either side of transcription start site of the gene. The paused promoter sequences (ranging 180–220 bp) were PCR-amplified (primers sequences available on request) and subcloned into a *pBSKS* vector containing a *lacZ* fragment to make a promoter-*lacZ* fusion. This promoter-*lacZ* fragment was then subcloned into the pCaSper-based enhancer-blocker vectors to get final constructs that were *w-prom/lacZ-IAB5-Casper* (Zhou et al. 1996; Ohtsuki and Levine 1998). To make the *w-IAB5-prom/lacZ* vector, the IAB5 enhancer was excised from the *w-prom-lacZ-IAB5-Casper* vector and cloned between *w* and *prom-lacZ* reporters. All of the cloned fragments were verified by sequencing.

Fly strain culturing and transgenics

All fly stocks were cultured at 25°C in standard cornmeal medium. The transgenic flies were created at BestGene, Inc., or were injected as per

standard protocol (Small et al. 1992). At least three independent lines were analyzed for each of the constructs for all the experiments (see Supplemental Table 1). For the crosses, virgin females of elongation factor mutants were crossed to males of the transgenic fly, followed by in situ hybridization. The elongation factor mutations were obtained from the Bloomington Stock Center, Exelixis, and Drosdel deletion project. The fly stocks used were *y¹ w¹¹¹⁸*, *Spt5^{MGE-3}/SM1*; *Psn¹⁴³/TM6_B* (*Spt5*), *y¹ w^{67c23}*, *Pn,Spt4^[k05316]/CyO* (*Spt4*), *y¹ w^{67c23}*, *P[w(+mC) y(+mDint2)] = EPgy2/rdx^{[EY06724]/TM3,Sb}* (*Nelf-E*), *c00768/TM6_B,Tb* (*Nelf-E*), *y¹*; and *ry506 P[*SUPor-P*]/NELF-A^{KG09483}/TM3, Sb¹ Ser¹ (*Nelf-A*)*.

In situ hybridization

All probe templates were obtained from PCR-amplified genomic fragments cloned into *pBluescript* vector (Fermentas) and confirmed by sequencing. PCR primers were designed by using Primer3 (<http://frodo.wi.mit.edu/cgi-bin/primer3/primer3-www.cgi>), and a list of primers used is available on request. For each template, antisense RNA probes were in vitro transcribed by using T7 or SP6 RNA polymerase and digoxigenin-UTP (Roche Molecular Biochemicals). Embryos were collected for 4 h and aged for an additional 2 h. Fixed embryos were hybridized with the riboprobes as described elsewhere (Jiang et al. 1991).

ChIP and qPCR

The ChIPs using RNA Pol II antibodies followed by qPCR were performed as described elsewhere (Chopra et al. 2009). In brief, a cocktail of Pol II antibodies 8WG16 and H14 was used for the ChIP assays on chromatin prepared from wild-type or mutant embryos.

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