Chromatin insulator and the promoter targeting sequence modulate the timing of long-range enhancer–promoter interactions in the Drosophila embryo

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The homeotic genes are essential to the patterning of the anterior–posterior axis along the developing Drosophila embryo. The expression timing and levels of these genes are crucial for the correct specification of segmental identity. The Abd-B (Abd-B) gene is first detected in the most posterior abdominal segments at high levels and gradually appears in progressively anterior abdominal segments in lower amounts. Regulatory mutations affecting this expression pattern produce homeotic transformations in the abdomen. The promoter targeting sequences (PTS) from Abd-B locus overcome the enhancer blocking effect of insulators and facilitate long-range enhancer–promoter interactions in transgenic flies (1, 2). In this study, we found that transgene activation by the IAB5 enhancer can be delayed by inserting a 9.5 kb 3' Abd-B regulatory region containing the Frontabdominal-8 (Fab-8) insulator and the PTS element. We found that the delay is caused by the PTS and an insulator, and it is not specific to the enhancer or the promoter tested. Based on these findings, we hypothesize that the delay of remote enhancers is responsible for the Abd-B expression pattern, which is at least in part due to the regulatory activities of the PTS elements and chromatin boundaries.

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Homeotic selector genes control segmental identities along anterior–posterior axis during Drosophila development. Each of these genes contains complex regulatory regions necessary for its expression patterns. The Abd-B (Abd-B) locus from the Drosophila bithorax gene complex consists of four downstream located, Parasegment (PS) specific regulatory domains, called infraabdominal (iab)-5, iab-6, iab-7, and iab-8, each of these controls Abd-B in one of the corresponding Parasegments from PS10 to PS13 to generate defined temporal and spatial gradient of Abd-B (Karch et al., 1985; Duncan, 1987; Mihaly et al., 1998; Sanchez-Herrero, 1991; Casanova et al., 1986; Celiker et al., 1990; Morata et al., 1986; Qian et al., 1991; Sanchez-Herrero et al., 1985). Abd-B is first expressed in the PS13 at stage 5 during embryogenesis and gradually appears in lower levels in more anterior segment during stages 9–11 (Harding and Levine, 1988). In the CNS, Abd-B protein exhibits a gradient with higher levels in posterior segments and lower levels in PS10 and 11 (Mihaly et al., 1997a). Genetic mutations inactivating one regulatory region will result in the expression of Abd-B in the affected segment to duplicate that of an immediate anterior segment. For example, an iab-7 mutation would result in PS12 to PS11 transformation producing a duplication of PS11 (Mihaly et al., 1998). To date, the exact mechanism of this transformation remains poorly understood.

Abd-B contains at least four classes of cis-regulatory elements responding to trans-regulators to orchestrate complex controls of Abd-B. At blastoderm stage, segmentation genes act on tissue specific enhancers such as IAB5, IAB7 and IAB8 (Sanchez-Herrero, 1991; Qian et al., 1991; Harding and Levine, 1988; Muller and Bienz, 1992; Shimell et al., 1994; Casares and Sanchez-Herrero, 1995; Estrada et al., 2002; Busturia and Bienz, 1993; Zhou et al., 1999; Bargueño et al., 2000). In late embryogenesis, the Polycomb group (PcG) and trithorax group (TrxG) genes respond to the activating and silencing signals of these early enhancers through dedicated Polycomb or trithorax response elements (PREs or TREs) and maintain the pattern of their activities throughout late development and into adult (Kennison, 1995; Paro et al., 1998; Bienz and Muller, 1995). Individual iab domains function autonomously, and are protected by domain boundary elements such as micastrad pigmental (MCP), front abdominal (Fab)-7 and Fab-8, which may prevent the spread of active or repressed chromatin (Mihaly et al., 1997a; Zhou et al., 1996, 1999; Bargueño et al., 2000; Gyurkovics et al., 1990; Galloni et al., 1993; Karch et al., 1994a; Hagstrom et al., 1996). Finally, a new class of cis-regulatory elements, the promoter targeting sequences (PTS) have been identified from the Abd-B locus (Zhou and Levine, 1999; Lin et al., 2003; Muller, 2000). The PTS overcomes the enhancer blocking effect of an insulator, and facilitates enhancer–promoter communication. Thus, PTS elements may play important roles in enhancer–promoter communications over long distances and intervening boundary elements in Abd-B.
Currently, progressively lower levels of Abd-B in segments anterior to PS13 and 14 have been explained by the presence of insulators or silencers, which may attenuate distal enhancers (Mihaly et al., 1998; Busturia and Bienz, 1993). However, no systematic studies were reported to explain the temporal difference of Abd-B expression in these segments. A “sequential opening” model hinted that different regulatory regions may become “open” at different time during development (Vázquez et al., 1993). However, Abd-B enhancers such as IAB5 from iab-5, IAB7 from iab-7 and IAB8 from iab-8 become active at precisely the same time during cellular blastoderm stage, making it difficult to imagine how these enhancers could end up “opening” the chromat of each iab at a different time point during embryogenesis (Figs. 1A–C) (Busturia and Bienz, 1993; Zhou et al., 1999; Barges et al., 2000). In the current study, we investigated whether the timing of enhancer–promoter interactions could generate the temporal and spatial gradient of Abd-B. For this purpose, we tested transgenic promoter activation by enhancers and regulatory regions from Abd-B. We found that the reporter gene lacZ activation by IAB5 can be delayed by inserting a 9.5 kb Abd-B 3′ regulatory region containing the Fab-8 insulator and the PTS (Hendrickson and Sakonju, 1995; Hopmann et al., 1995). A similar delay of enhancers can be reproduced by simply inserting an insulator and the PTS. We hypothesize that the process of overcoming insulators by the PTS delays enhancer–promoter interaction. 

Results

Early enhancers from Abd-B become active at the beginning of zygotic transcription

The early embryonic enhancers from the Abd-B locus, IAB5, IAB7 and IAB8, are regulated by gap genes and segmentation genes, which in principle are able to activate transcription as soon as zygotic transcription starts during cellularization (Busturia and Bienz, 1993; Zhou et al., 1996, 1999). As can be seen in Figs. 1A–C, this is indeed the case when each of them is linked to the lacZ reporter in transgenic embryos. There is no detectable temporal difference in activating the lacZ gene among these enhancers. However, it is not clear whether these enhancers are able to activate transcription from the endogenous location at the same time as well. To test this, we compared the activity of the lacZ transgene from a P-element insertion (fs05369) into the iab-7 region (Zhou and Levine, 1999). The P-element in (fs05369) is inserted about 2 kb away from the identified IAB7 enhancer and there is no insulator or PTS element located between IAB7 and the transgenic lacZ promoter. When the lacZ gene from the P-element was examined, it was detected in PS12 and 14 in the early blastoderm stage, in a pattern similar to that of the IAB7 enhancer (Fig. 1D). This result suggests that enhancers in the Abd-B locus become active at the onset of zygotic transcription. There is no difference in the onset of these enhancers. In addition, these enhancers remain at similar strength until stage 10 before diminishing (Figs. 2A–C).

A 9.5-kb regulatory region of Abd-B delays the IAB5 enhancer

Contrary to the simultaneous activation of early enhancers from Abd-B, the Abd-B protein is expressed in a temporal gradient, which appears first in the most posterior segments and gradually becomes detectable in more anterior segments (Harding and Levine, 1988). Similarly, in situ hybridization shows that Abd-B transcript first appears in ps13 around stage 5 of embryogenesis (Fig. 2D). It is detectable in PS13 and PS14 by stage 9 (Fig. 2E). Abd-B expression in PS12 appears at early stage 10, followed by expression in PS11 and later on in PS10 (Fig. 2F). Thus, there is a significant delay between the activation of Abd-B enhancer and the actual expression of Abd-B mRNA in PS10–12. Specifically, while IAB5 and IAB7 become active in PS10 and PS12 in early embryogenesis (stage 5 Figs. 1A, B), the expression of Abd-B in these segments is not seen until stage 10, about 3 h later (Fig. 2F). This result suggests that there is a delay in the productive interactions between these enhancers and the Abd-B promoter.

There are at least two alternative explanations to the delayed expression of Abd-B in more anterior abdominal segments. First, the activation of the corresponding regulatory region is temporally regulated. For example, iab-8 is activated first, while iab-5 is activated later. However, there is no evidence supporting this possibility, and the results from Fig. 1 suggest that there is no timing difference in the activation of these regulatory domains. We propose an alternative hypothesis that enhancer–promoter interactions within the Abd-B
gene are delayed by regulatory elements such as the PTS and Fab insulators.

To test whether IAB5 could be delayed, we constructed a transgene complex containing \( w \) and eveGFP (Lin et al., 2004) genes, the IAB5 enhancer, and the 9.5-kb regulatory region from the 3' of Abd-B inserted between IAB5 and eveGFP. The 9.5-kb DNA is from a region called transvection mediating region (tmr), which is known to mediate long-range enhancer–promoter interactions in the endogenous locus (Hendrickson and Sakonju, 1995; Hopmann et al., 1995; Sipos et al., 1998). This region contains multiple cis-elements including the Fab-8 insulator, the PTS, a PRE, the IAB8 and IAB7 enhancers (Zhou and Levine, 1999; Zhou et al., 1999; Barges et al., 2000). The tmr was inserted in the orientation given in Fig. 3, which makes the relative positioning of different DNA elements similar to that of the endogenous arrangement (Fig. 7A).

eveGFP expression is first activated by the promoter-proximal located IAB8 at stage 5 as a single band in the posterior region of the embryo (Fig. 3A). The IAB5 enhancer, on the other hand, is barely detectable at this stage (Fig. 3A, arrow). Its activity becomes evident but significantly weaker than that of IAB8 at stage 6, the beginning of gastrulation (see arrow in Fig. 3B). As development proceeds, IAB5 intensifies, and by stage 8 (4-h-old embryos), it is as strong as IAB8 (Fig. 3C). The IAB7 enhancer overlaps with the second and third band of IAB5, however, it is known to be very weak in transgenic embryos (Zhou et al., 1999), and is too weak to be seen when overlapped with IAB5. This is probably due to the fact that the IAB7 in the 9.5-kb DNA represent only a partial enhancer from \( iab-7 \) region. Compared with IAB8, IAB5 must overcome the enhancer-blocking effect of the Fab-8 insulator to activate transcription. And based on our previous studies, the IAB5–eveGFP interaction is due to the anti-insulator activity of the PTS, which is also present in the tmr (Zhou and Levine, 1999; Zhou et al., 1999). Thus this result strongly suggests that the IAB5 enhancer–eveGFP gene promoter gene interaction is delayed. The enhancer delay hypothesis is also supported by comparing IAB5

![Fig. 2. Abd-B expression during embryogenesis. (A–C) IAB5, 7 and 8 enhancer activities in stage 10. These embryos carry the same transgenes as in Figs. 1A–C. (D–F) Abd-B expression in stages 5, 10, and 11. It first appeared in PS13 and occupies PS13 and 14 by stage 9. In stage 10 Abd-B becomes detectable in PS10–12 with sequential delay from PS12 to PS10. D. Stage 5 embryos, showing the initial expression of Abd-B in PS13. E. In stage 9, Abd-B expression is seen in PS13 and PS14. (F) Abd-B is expressed in PS10–14 in stage 10. A comparison of Abd-B expression in these stages with lacZ activation by IAB enhancers indicates delayed of Abd-B activation in PS10–12.](image1)

![Fig. 3. The tmr region from Abd-B delays the IAB5 enhancer activity. eveGFP expression in stage 5, 6 and 8 embryos. The transgene contains a 9.5 kb tmr sequence from the 3' of Abd-B, which includes a proximal IAB8 enhancer, the Fab-8 insulator, the PTS and the distal IAB7 enhancer. IAB5 is approximately 14 kb away from eveGFP, where as IAB8 and IAB7 are 5 and 11 kb away, respectively. (A) Stage 5 embryos showing IAB8 expression (posterior band) and trace amount of IAB5 activity (arrow). (B) A gastrulating embryo showing elevated IAB5 activity (arrow), which is still weaker than that of IAB8 (posterior solid band). (C) Stage 8 embryos showing stronger IAB5 activity. Compared to that of IAB8 (PS13), IAB5 activity is at least as strong, if not stronger (see PS10).](image2)
activity in Figs. 1A, 2A with that in Fig. 3. The former shows strong activation of lacZ early on and the activity persist until stage 10 when it starts to decline, while the latter shows a gradual increase of expression, which peak at stage 10. Thus the selective delay of IAB5 enhancer suggests that the Fab-8 insulator and the PTS element from the tmr affect the timing of IAB5-eveGFP interaction, as these are the only DNA elements from this region known to affect enhancer–promoter interactions.

The PTS delays enhancer–promoter interactions

Our previous studies indicated that the PTS has an anti-insulator function, in that when an insulator is present between an enhancer and a promoter, the PTS could bypass the insulator and allow the enhancer to activate the promoter (Zhou and Levine, 1999). In addition, the PTS facilitates the targeted enhancer and restricts it to a single promoter (Lin et al., 2003). Furthermore, the PTS requires the presence of an insulator, without which the PTS does not affect either the promoter or the promoter (Lin et al., 2004). To test whether the PTS-mediated anti-insulator activity could delay promoter activation, we inserted the 340 bp suHw insulator (Dorsett, 1993; Cai and Levine, 1995; Scott and Geyer, 1995) and the 625 bp PTS (Zhou and Levine, 1999) between the 1.6 kb IAB8 enhancer (Zhou et al., 1999) and the 3′ of lacZ. As a control for enhancer timing, we also inserted a 300 bp NEE enhancer from the rhomboid gene at the 5′ of the lacZ promoter (Ip et al., 1992). Based on our previous studies, the NEE enhancer when placed near the lacZ promoter will not be regulated by the insulator and the PTS located at the 3′ end of lacZ 3′ (Lin et al., 2004).

We selected lacZ promoter-targeted strains and compared the staining patterns of embryos at different time points of the cellular blastoderm stage (stage 5, see Figs. 4A–D). Staging of the embryos is done by examining the degree of cellularization. Stage 5 embryos undergo cellularization that last from 2 h 30' to 3 h 15' old embryos. At early stage 5, the beginning of cellularization, where intercellular membrane are growing, NEE strongly activates lacZ, while IAB8 activity is barely detectable (Fig. 4A). When the embryos complete cellularization, as marked by the clear demarcation between the base of the cells and the yolk, we see strong activation of lacZ by both enhancers. During cell elongation phase at late stage 5, however, IAB8 continually grows stronger while NEE remains at the same strength. The stronger IAB8 also leads to ectopic activation of lacZ in the anterior region of the embryo (Fig. 4C) (Zhou and Levine, 1999). The activities of the two enhancers remain stable at the end of stage 5 (Fig. 4D). In a separate control experiment, we monitored IAB8 activity when the enhancer is located alone at the 3′ end of the lacZ (W89) (Lin et al., 2004). We found weak activation of lacZ by IAB8 throughout stage 5 with no obvious changes of activity levels at similar time points shown (Figs. 4E–H). These results suggest that the selective delay of IAB8 activity in Figs. 4A–D is mainly due to the PTS and suHw. These results also confirm our previous demonstration that PTS facilitates long-range enhancer–promoter interactions (Lin et al., 2003).

Although PTS and insulator delays enhancers, the large distances between enhancer and promoters in both the endogenous locus and
the transgenic construct in Fig. 3 could also delay the enhancer. For this reason, we attempted to test whether the delay seen in Fig. 3 is in part due to long enhancer–promoter distance. We inserted a 6 kb λ spacer between the 3’ of lacZ and IAB5 in a transgene described previously (Lin et al., 2004), separating the IAB5 enhancer from the promoter by approximately 10 kb. However, IAB5 becomes undetectable from this distance in the absence of the PTS (data not shown). A similar experiment with the NEE enhancer in construct HN (Lin et al., 2004) also reduced the enhancer activity to a level beyond the sensitivity of in situ hybridization. Thus, it is not possible to analyze the role of large distances (over 10 kb) in enhancer delay in transgenic embryos. Nevertheless, when we compared the activities of IAB5 at a location just next to the promoter and at a location at the 3’ end of lacZ, about 4.5 kb away, we could not discern any timing differences from these two locations (data not shown).

Enhancer delay is independent of the identities of the enhancer and insulator and it requires the presence of both the PTS and an insulator.

We next determined whether enhancer delay is specific to Abd-B enhancers. We placed the 300 bp NEE enhancer at 3’ of lacZ and a 228 bp H1 enhancer from the hairy gene (Riddihough and Ish-Horowicz, 1991) at the 5’ of lacZ. When a control spacer 1.6 kb λ DNA is included in the transgene to control for distance, the activity pattern of the two enhancers did not change during stage 5 of embryogenesis: H1 activates strong lacZ expression throughout the period, while NEE directs robust lacZ activation with similar levels in all three time points (Figs. 5A–C). When the spacer was replaced by the suHw insulator, NEE is selectively blocked (Figs. 5D–F). But when the PTS is also inserted into the transgene, it overcomes the suHw insulator and targets NEE to the lacZ promoter. Interestingly, lacZ activation by NEE is selectively delayed at the beginning of stage 5 (Fig. 5G, about 2 h 30 min after fertilization), while H1 strongly activates lacZ. NEE is barely detectable from most embryos. In mid stage 5 (Fig. 5H, about 2 h 45 min old), NEE first becomes detectable with low levels. High level of NEE activity is only detected after mid and at late stage 5 (Fig. 5I, about 3-h-old embryos). This result suggests that the anti-insulator activity of PTS results in enhancer delay, and enhancer delay is not specific to Abd-B enhancers, or the insulator used.

We also tested whether PTS alone could delay a distal enhancer. Our previous study indicated that promoter targeting by PTS requires the presence of an insulator, which must be placed between an enhancer and a promoter. Inserting the PTS alone in the transgene does not cause promoter targeting (Lin et al., 2004). To test whether PTS alone affects the timing of enhancers, we inserted the 625 bp PTS between the 3’ of lacZ and the distal NEE enhancer. When three different time points during stage 5 were examined, NEE activates reporter gene w without significant difference in activity levels among the three time points (Figs. 6D–F). The expression of w is very similar to control transgene expression when the 1.6 kb λ spacer is inserted (Figs. 6A–C). This result suggests that the PTS does not alter the timing of enhancer–promoter interactions in the absence of an insulator. Since PTS alone does not cause promoter targeting, or exhibit anti-insulator activity, we conclude that enhancer delay is a property of anti-insulator and promoter targeting functions of the PTS and requires the presence of both insulator and the PTS. 

If overcoming the enhancer blocking effect of an insulator delays enhancer–promoter interaction, then adding more insulators may increase enhancer delay. To test this hypothesis, we inserted the 1.6 kb DNA from the tmr containing fab-8 and PTS (Zhou and Levine, 1999) at two places of the transgene, one between the 3’ of lacZ and the other between H1 and w promoter. Most transgenic strains exhibit promoter targeting to the w promoter. A representative line is shown in Figs. 6G–I. Similar to Figs. 5G–I, the NEE enhancer is delayed as little or no NEE could be detected in early to mid stage 5. Peak NEE activity is only seen in stage 6 embryos when gastrulation has begun (note the cephalic furrow formation), a full developmental stage later than the peak level in Fig. 5I when one insulator/PTS pair is inserted (Fig. 6I). In embryos carrying the control transgene at a similar stage, the NEE level has begun to decrease at this stage (see Fig. 6C). This
result further supports the model that enhancer delay is mainly due to the anti-insulator activity from the PTS element. It also suggests that in the Abd-B locus remote enhancers such as IAB5 would be delayed a longer time than enhancers that are located relatively closer, for example IAB7.

**Discussion**

Specific temporal and spatial gradients of Hox gene expression are essential for the proper specification of the anterior-posterior axis of the body plan during development. Mutations affecting the regulatory regions for the Hox genes have been shown to alter the timing of Hox gene expression and result in developmental defects both in mouse and flies (Hagstrom et al., 1996; Juan and Ruddle, 2003; Mihaly et al., 1997). The Abd-B gene from the Drosophila bithorax complex is activated first in the most posterior segments PS13 and PS14, followed by the sequentially delayed activation in PS12, PS11 and PS10 (Harding and Levine, 1988; Galloni et al., 1993; Celinker et al., 1989; Boulet et al., 1991). To date, no systematic study has been conducted to address the mechanism of this gradient, nor are there proposals explicitly trying to explain the phenomenon. It is possible that the chromatin of different iab domains are sequentially activated over time in an Abd-B proximal to distal direction (Vazquez et al., 1993), in a manner similar to the temporal collinear activation of the Hox gene cluster (Pourquie, 1998; Soshnikova and Duboule, 2009). However, enhancers identified from these iabs including IAB5, IAB7 and IAB8 are activated precisely at the same time in cellular blastoderm stage embryo when segmentation genes are active (Fig. 1). In particular, endogenous IAB7 activity can be detected in blastoderm stage embryos, but Abd-B is not detected in PS12 until early stage 10, about 3 h later (compare Figs. 1D and 2F). These results strongly suggest that the delayed Abd-B expression in more anterior abdominal segments is due to the delay in enhancer–promoter interaction. Consistent with this model, we have demonstrated that inserting a 9.5-kb tmr sequence from the 3′ region of Abd-B containing an insulator and a PTS significantly delayed the timing of transgene activation by IAB5 (Figs. 3A–C). We further demonstrated that the delay is mainly caused by insulator and the PTS, and the delay is not specific to Abd-B enhancers, as a heterologous NEE enhancer can also be delayed (Figs. 5 and 6). We also found that enhancer delay depends on the presence of an insulator inserted between the enhancer and the promoter, and increasing the number of insulators prolongs the delay. This study strongly suggests that overcoming the enhancer-blocking effect of insulators in the endogenous locus contributes at least in part to the temporal delay of distal Abd-B enhancers. In addition, it is possible that overcoming multiple insulators may contribute a longer delay for the more remote enhancers such as IAB5.

These analyses support an enhancer-timing model that accounts for, at least in part, the temporal and spatial gradient of Abd-B expression. We propose that domain boundaries and PTS elements from Abd-B regulate long-range enhancer–promoter interactions by establishing stable interactions between the Abd-B promoter and its regulatory domains (iabs) (Lin et al., 2003). The stable interaction facilitates the activities of these distant enhancers, but in the process also delays promoter activation by these enhancers. Such interactions may require the assembly of complex structures linking the enhancer and its promoter, and/or involve complex movement of chromatin loops, processes that need significant longer time than that of a simple enhancer–promoter interaction. As a result, enhancers from iab-5 to iab-7 are significantly delayed (Fig. 7A). We also propose that a specific boundary and the nearby PTS mainly delay the enhancer they regulate. For example, Fab-8 and the linked PTS mainly delays IAB7.

Enhancer-timing model could in part explain the spatial gradient of Abd-B. Since the delay of distal enhancers reduce the amount of time Abd-B protein accumulates in a specific segment, there would be high levels of Abd-B in PS13, but gradually lower levels in PS12, PS11 and PS10. However, this model does not exclude other possibilities such as distance and enhancer strength may play in generating the expression gradient. It is possible that long distance may both reduce enhancer strength and delay enhancer–promoter interactions, which
explains the fact that IAB5 is further delayed compared to IAB7. We were unable to test the role of distance in delaying enhancer–promoter interaction because most embryonic enhancers are sensitive to distances and become undetectable when located at about 10 kb away from the promoter. When a shorter distance of 5 kb was tested, no obvious delay could be detected (data not shown).

The enhancer delay model is compatible with the behavior of dominant gain of function mutations due to deletion of boundary elements from the 3′ of Abd-B. For example, the Fab-7 deletions cause the expression of Abd-B in PS11 to elevate to that in PS12 and homeotic transformation of PS11 into a copy of PS12 (Mihaly et al., 1997a; Hagstrom et al., 1996). This could be due to the loss of an enhancer delay normally in place when Fab-7 is present. Its deletion allowed the two domains to fuse as one, thus, enhancer from iab-6 become regulated the same way as an iab-7 enhancer (IAB7) (Mihaly et al., 1997a). Consequently, both enhancers arrive at the promoter at about the same time, and activate the same amount of Abd-B transcription in both segments (Fig. 7C). Finally, the enhancer-timing model is compatible with the collinear arrangement of Abd-B regulatory domains. For example, to express high levels of Abd-B early in PS13, the IAB8 enhancer must be placed closer to the promoter. In contrast, to produce a lower level and delayed Abd-B expression in PS10, the enhancer IAB5 must be placed far away, and separated by multiple insulators and PTS elements to delay its interaction with the Abd-B promoter.

Materials and methods

Transgenic plasmid construction

Constructs for Figs. 1–3

Control transgenes with single enhancers IAB5, IAB7, IAB8 and NEE was made by inserting these enhancer elements into the BamHI site at the 5′ of Tp-lacZ gene in C4PLZ. To make GFP-tmr construct, the lacZ gene from CasN was replaced by a eveGFP fusion gene that contains the 250 bp eve basal promoter from −42eveCaSper (Small et al., 1992), 900 bp GFP gene and 500 bp SV40 3′ UTR (from Invitrogen). The 1.0 kb IAB5 enhancer (Busturia and Bienz, 1993) is cloned into the PstI site and the 9.5 kb tmr (Zhou et al., 1999) was inserted into NotI site.

Constructs for Fig. 4

W89, a 1.6 kb PstI fragment is inserted at the PstI site of C4PLZ located at the 5′ of lacZ gene in C4PLZ. To make GFP-tmr construct, the lacZ gene from CasN was replaced by an eveGFP fusion gene that contains the 250 bp eve basal promoter from −42eveCaSper (Small et al., 1992), 900 bp GFP gene and 500 bp SV40 3′ UTR (from Invitrogen). The 1.0 kb IAB5 enhancer (Busturia and Bienz, 1993) is cloned into the PstI site and the 9.5 kb tmr (Zhou et al., 1999) was inserted into NotI site.

Constructs for Fig. 5

HZAN was made by placing at 228 bp Spe I-BamHI fragment of the HI enhancer (Zhou et al., 1996) between the w and lacZ promoters,
adding a blunt 300 bp fragment of the NEE enhancer (Zhou et al., 1996) at the Stul site at the 3′ of lacZ, and inserting a 1.6 kb spacer sequence at the BgIII site between the 3′ of lacZ and the NEE (Zhou et al., 1996). The construct HSN under Figs. 3D–F was made by inserting a BamHI-BgIII fragment of suHw insulator at the BgIII site of HZN vector. W250 was made by adding the 625 BamHI-BgIII fragment of Pts at the regenerated BgIII site of this vector.

Constructs for Fig. 6

The PTS in HZN was made by inserting the 625 bp BamHI-BgIII PTS insert into the BgIII site of HZN vector. HZM2MN was made by inserting the 1.7 kb BamHI fragment from the ttr containing both Fab-8 and PTS (Zhou and Levine, 1999) into the BgIII site of HZN vector first, and then inserting another copy at the BamHI site at the 5′ of lacZ.

P-element transformation and genetic crosses

P-element transformation vectors containing the mini white gene were introduced into the w1118 fly embryos by microinjection as described previously (Rubin and Spradling, 1982). For each of the transgenic constructs, 10 to 20 independent transformants were selected and maintained as transgenic stocks. P-element insertion into the region fs(3)03569 was obtained from the Bloomington Stock Center (BL302).

RNA in situ hybridization

Whole mount RNA in situ hybridization was performed as described previously (Zhou and Levine, 1999; Tautz and Pfeifle, 1989). Briefly, staged embryos were collected, dechorionated with 50% bleach for 5 min, and fixed with 4% formaldehyde in PBS and for 30 min. Methanol was added to the fixed embryos to remove the vitelline membranes. These embryos were incubated with pre-hybridization buffer (50% formamide, 4XSSC, 1X Denhardts, 20μg/ml RNA, 250μg/ml ssDNA, 50 μg/ml heparin, 0.1% Tween 20 and 5% dextran sulfate) at 55 °C for 1 h and 12 μl of final pre-hybridization buffer (50% formamide, 4XSSC, 1X Denhardts, 50 μg/ml herring sperm DNA) was added to each embryo. Anti dig-AP antibody (Roche) was added to the embryo at 1:2000 dilution. Color reaction was carried out at room temperature with AP staining buffer containing NBT and BCIP (Roche). Stained embryos were rinsed, fixed with ethanol, and mounted on glass slides.

Staging of Drosophila embryos

Embryo staging was done according to D. B. Robert, 1998, Drosophila, A practical Approach (pp 186-197). Briefly, we collect, fix approximately 700 2- to 6-h and 300 4- to 8-h embryos and conduct in situ hybridization. We stage embryos based on visual examination of the degree of cellularization and other developmental features including cephalic furrow formation and germ band extension. Stage 5 embryogenesis lasts 45 min, which contains early stage 5 from 2 h 30′ to 2 h 45′, where cell membrane starts grow, mid stage 5, from 2 h 45′ to 3 h, when cell membrane is fully formed, and late stage 5, from 3 h to 3 h 15′, when cells start to elongate. We observe at least 30 embryos for each developmental stage. The expression of the transgenes was found to be consistent and representative embryos were chosen.

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