Enhancer-PRE communication contributes to the expansion of gene expression domains in proliferating primordia

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SUMMARY
Trithorax-group and Polycomb-group proteins interact with chromosomal elements, termed PRE/TREs, to ensure stable heritable maintenance of the transcriptional state of nearby genes. Regulatory elements that bind both groups of proteins are termed maintenance elements (MEs). Some of these MEs maintain the initial activated transcriptional state of a nearby reporter gene through several rounds of mitosis during development. Here, we show that expression of hedgehog in the posterior compartment of the Drosophila wing results from the communication between a previously defined ME and a nearby cis-regulatory element termed the C enhancer. The C enhancer integrates the activities of the Notch and Hedgehog signalling pathways and, from the early wing primordium stage, drives expression to a thin stripe in the posterior compartment that corresponds to the dorsal-ventral compartment boundary. The ME maintains the initial activated transcriptional state conferred by the C enhancer and contributes to the expansion, by growth, of its expression domain throughout the posterior compartment. Communication between the ME and the C enhancer also contributes to repression of gene expression in anterior cells. Most interestingly, we present evidence that enhancers and MEs of different genes are interchangeable modules whose communication is involved in restricting and expanding the domains of gene expression. Our results emphasize the modular role of MEs in regulation of gene expression within growing tissues.

KEY WORDS: hedgehog, Epigenetic inheritance, Maintenance element, Notch, Ci, Drosophila

INTRODUCTION
Gene expression is generally governed by cis-regulatory elements that integrate cell-type and temporal information to generate accurate and stereotyped patterns of expression in space and time (Levine, 2010). One class of regulatory modules is embryonic enhancers, which drive gene expression as a result of transcription factor binding at the enhancer sequence. Very frequently, these transcription factors are only transiently expressed during development and the gene expression state of their target genes is maintained by the Polycomb group (PcG) and the Trithorax group (TrxG) of proteins. These form the basis of a cellular memory system that maintains the transcriptional state of the target genes heritable during development (for reviews, see Muller and Kassis, 2006; Schwartz and Pirrotta, 2007). The genes controlled by the PcG/TrxG system have PcG and TrxG response elements (PRE/TREs), to which these proteins bind and keep the gene either permanently repressed (PcG) or active (TrxG). In order to reflect the dual function of these regulatory elements that bind both groups of proteins, PRE/TREs are termed maintenance elements (MEs) (Brock and van Lohuizen, 2001). Some of these elements have been shown to maintain the initial activated transcriptional state of a nearby reporter gene through several rounds of mitosis during development (Cavalli and Paro, 1998). This is the case for Drosophila Hedgehog (Hh), a signalling molecule expressed in the posterior (P) compartment of all limb primordia and involved in anterior-posterior axis formation (Basler and Struhl, 1994; Tabata et al., 1992). An ME situated upstream of the hh transcription start site is able to maintain Gal4-driven lacZ reporter gene expression through several rounds of mitosis (Bejarano and Milan, 2009; Mauange and Paro, 2002).

Both genetic and epigenetic mechanisms are involved in regulation of hh expression in the Drosophila wing (Fig. 1A). The transcription factor Ci is expressed in the anterior (A) compartment, where it is required to repress hh expression (Apidianakis et al., 2001; Bejarano et al., 2007; Méthot and Basler, 1999), and the homeodomain proteins Engrailed (En) and Invected are expressed in the posterior (P) compartment, where they are required to repress Ci expression (Eaton and Kornberg, 1990) and relieve Ci-mediated repression of hh (Bejarano and Milan, 2009). PcG proteins help to maintain the repression of hh expression in A cells whereas TrxG proteins contribute to maintaining the expression of hh in P cells (Bejarano and Milan, 2009; Chanhas and Maschat, 2005). Notch activity at the dorsal-ventral (DV) compartment boundary has also been reported to participate in the regulation of hh expression; however, the mechanistic basis behind this is uncertain (Bejarano and Milan, 2009). Although communication between the hh-ME and those cis-enhancers that integrate positional information conferred by the activities of Ci, En and Notch has been proposed as a way to initiate and maintain the transcriptional state of hh in P cells, the identity of these enhancers and the proposed communication remain elusive.

Here, we have isolated a 4.3-kb cis-regulatory region that recapitulates hh expression in the P compartment of the wing primordium. This fragment includes the previously defined hh-ME and a nearby enhancer (termed C enhancer) that responds to En, Ci and Notch and drives gene expression to a thin stripe in the P compartment that corresponds to the DV compartment boundary. We present evidence that the ME maintains the initial transcriptional

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activated state conferred by the C enhancer and contributes to expansion, by growth, of the expression domain throughout the P compartment. Communication between the ME and the C enhancer also contributes to repression of gene expression in A cells. Most importantly, we show that enhancers and MEs of different genes are interchangeable modules whose communication is involved in the expansion and repression of gene expression in the *Drosophila* wing. These results emphasize the modular role of MEs in regulating gene expression within growing tissues.

**MATERIALS AND METHODS**

*Drosophila* strains

*Df(2R)ren[E]* deletes both engrailed and invected (Gustavson et al., 1996); *Su(z)2H* (Wu and Howe, 1995); *UAS-CyZ [CyZ1A*, a truncation of Cy at amino acid residue 975 that behaves as a constitutive transcriptional repressor (Méthot and Basler, 1999)]; vg-GAL4 (Williams et al., 1994); UAS-NotR (Presente et al., 2002); apoe4, brm2, trx5B, grvK44, UAS-en, UAS-lacZ, UAS-GFP, UAS-NINTRA and hhP30 (hh-lacZ in the text) are described in FlyBase.

**Antibodies**

Rat anti-Ci (Motzny and Holmgren, 1995), rabbit anti-βGal (Cappel), rabbit anti-GFP (Upstate), mouse anti-Pc [Apa 1 (Capdevila et al., 1994)], mouse anti-En [4D9 (Pate1 et al., 1989)], mouse anti-Wg [4D4 (Brook and Cohen, 1996)], mouse anti-Notch [C458.2H (Diederich et al., 1994)], mouse anti-Cut (2B10) and rabbit anti-βGal (40-1A) are described in the Development Studies Hybridoma Bank.

**Genetic mosaics**

The following *Drosophila* genotypes were used to generate loss-of-function clones (Xu and Rubin, 1993):

- hs-FLP; FRT42B *Df(2R)ren[E] Su(z)2H* / FRT42B Ubi.GFP; hh-lacZ/+; hs-FLP; FRT42B *Df(2R)ren[E] Su(z)2H* / FRT42B Ubi.GFP: ABC-lacZ/+.

Clone induction by heat-shock was carried out 2-4 days before wing disc dissection.

**Molecular characterization of the Mrt allele of hh**

A collection of primers were used to amplify and sequence the whole 4.3-kb genomic region upstream of the hh transcription start site from wild-type and hhMrt homozygous larvae. Primers (5'-3') used to amplify the region containing the deletion in the hhMrt allele were:

Hh-3Kb-Top: GGCGTCTCCGTCTGCTTTTAATTTC; Hh-3Kb-Bot: ATACGACCTTTTATTTACCTTA.

The hhMrt deletion maps to the following BDGP Release 5/dm3 assembly coordinates: chr3R: 18970938-18970961.

**Reporter constructs of hh**

Reporter genes were built from the hs43-nuc-lacZ vector, which contains the minimal (TATA box) promoter from the *hs34* gene (Estella et al., 2008). The A, B and C modules spanning the 4.3-kb genomic region upstream of the hh-RB transcription start site (according to FlyBase), and excluding the hh promoter, map to the following FlyBase sequence locations:


The predicted hh promoter maps to the following FlyBase sequence location: chr3R: 18967684-18967675.

For **ABC-lacZ** and **ABC3H**-lacZ, a 4.3-kb fragment upstream of the hh transcription start site was amplified by PCR from genomic DNA from wild-type and hhMrt homozygous larvae using the following primers (5'-3'):

ModABC-Fwd: ACACGACACACACACTAGCCTCGAGTTC; ModABC-Rev: TAAAGTACTCTGGAATATACATAAG.

The corresponding PCR products were cloned into the pTZ57R vector (Fermentas), then excised from this vector using XbaI and BamHI and subsequently cloned via the SpeI and BamHI sites into the phs43n-nuc-lacZ vector.

For **A-lacZ**, **B-lacZ**, **C-lacZ** and **C3H**-lacZ, in order to amplify by PCR the A, B and C modules from genomic DNA of wild-type or hhMrt homozygous larvae, the following primers (5'-3') were used:

ModA-Fwd: GGAATTCACACCGCACACACACTACTAC; ModA-Rev: ATTTGCGGCGCTTTTTAACTCTTTTCTTGAT; ModB-Fwd: GGAATTCATAATGATACGAGAAAAAAG; ModB-Rev: ATTTGCGGCGCAACATATAAAGCTTGCAAGCCA; ModC-Fwd: GGGGTACCAAGTCTTTTGCCTGT; ModC-Rev: TTGGTACGCTTTTATTGCCATGA.

PCR fragments containing modules A and B were digested with EcoRI and NotI, the fragment containing the module C was digested with KpnI and NotI, and subsequently cloned into the phs43n-nuc-lacZ vector (Estella et al., 2008).

For **AB-lacZ** and **BC-lacZ**, module AB was amplified by PCR from genomic DNA of wild-type larvae using ModA-Fwd and ModB-Rev primers and module BC was amplified by PCR using ModB-Fwd and ModC-Rev primers. Both fragments were digested with EcoRI and NotI and cloned into the phs43n-nuc-lacZ vector.

For **AC-lacZ** and **ACM3**-lacZ, module C was amplified by PCR from genomic DNA of wild-type and hhMrt homozygous larvae with the following primers (5'-3'):

ModAC-Fwd: ATTTGGGCGCGAAGTCTTTTGTGCTG; ModAC-Rev: TTGGATCCTAATTTTTTTTTTCTACATGA.

The fragment was digested with NotI and BamHI and cloned downstream of ModA in the phs43n-nuc-lacZ vector.

For **Ak-lacZ**, a 650-bp fragment from Lambda (λ) Phage was amplified by PCR with the following primers (5'-3'):

Lambda-Fwd: ATAAAGACTAGCGGCCGCAATGAAATACAGTCCT; Lambda-Rev: ATAAAGACTAGCGGCCGCTGACTAAGTCA.

The PCR product was digested with EcoRI and cloned upstream of ModB in the phs43n-lacZ vector.

For **AB-lacZ**, module AC was amplified by PCR from **AC-lacZ** with the following primers (5'-3'):

ModA-Fwd: GGAATTCACACCGCACACACACATAC; ModB-Rev: TGGATCCTAATTTTTTTTTTCTACATGA.

The PCR product was digested with EcoRI and cloned upstream of ModB in the phs43n-lacZ vector.

For **A-vgBE-lacZ**, module A was digested from the **ModAC-lacZ** with EcoRI and NotI; vgBE was digested from vgBE-lacZ with NotI and BamHI. Both fragments were ligated into the phs43n-lacZ vector digested with EcoRI and BamHI.

For **iab-7-BC-lacZ**, the iab-7 PRE region (chr3R: 12725498-12726604) regulating Abd-B was amplified by PCR with the following primers (5'-3'):

iab-7-Fwd: GATGCTATCGGAGTGTGAATGTTGATG; iab-7-Rev: CCGATTTCGCTGACGAGT.

The 1120-bp PCR fragment was cloned in the pCR-XL-TOPO vector (Invitrogen), then excised from this vector with EcoRI and cloned upstream of module BC in the phs43n-nuc-lacZ vector. FlyBase coordinates of PCR primers are described in Table S1B in the supplementary material.

**Reporter constructs of vg**

Reporter genes were built from the hs43-nuc-lacZ vector, which contains the minimal (TATA box) promoter from the *hs34* gene (Estella et al., 2008). The vgBE-lacZ reporter was generated by amplifying the vestigial BE region (Kim et al., 1995) by PCR from genomic DNA (chr2R: 8776380-8777133) from wild-type larvae, using the following primers (5'-3'):

vgBE-Fwd: GGGCAACGCGGCCGCAAGTCTTTTGTGCTG; vgBE-Rev: ATTTGCGGCGCAACATATAAAGCTTGCAAGCCA.

The vgBE PCR product was digested with EcoRI and BamHI and cloned into the phs43n-lacZ vector.

The vgBE PCR product was digested with NotI/BamHI and cloned into the phs43n-lacZ vector.
The vgPRE-lacZ reporter was made by amplifying the predicted PRE region of vestigial (chr2R:8793522–8790996) by PCR using the following primers (5’-3’):

vgPRE Fwd ATTTGGATCCCTATGCGATTAATAAGTTTGAATGG;
vgPRE Rev TCTACAGGATCCGAACTTGGCAACCTTATGCC.

The vgPRE PCR product was digested with BamHI and introduced into the pHs43n-lacZ vector.

The vgBE-PRE2-lacZ reporter was generated by introducing the vgPRE PCR product (digested with BamHI) into the BamHI site of the pHs43n-vgBE-lacZ construct.

The vgBE (Kim et al., 1995) and vgPRE (Schuettengruber et al., 2007) regions map to the following FlyBase sequence locations:

vgBE: chr2R: 8776380–8777132;
vgPRE: chr2R: 8790966–8793542.

FlyBase coordinates of PCR primers are described in Table S1B in the supplementary material.

Bioinformatic identification of Ci and Su(H) motifs

With the predictive models published in the literature for Su(H) and Ci/Gli consensus binding sites (Bailey and Posakony, 1995; Kinzler and Vogelstein, 1990; Lecourtois and Schweisguth, 1995), we used the MatScan program (Blanco et al., 2006) to obtain a list of putative Ci and Su(H) binding sites (up to two changes in the consensus) on the C module of hh (BDGP Release 5/dm3 assembly, coordinates: chr3R:18970798–18971949). We filtered out the predictions that were not conserved in at least five species (including Drosophila pseudoobscura or more distant species) in the multiple alignments of Drosophilids (UCSC Conservation track) (Fujita et al., 2011).

RESULTS

ABC, a 4.3-kb region that recapitulates the expression of hh in Drosophila tissues

We used a transgenic reporter gene assay to search for hh cis-regulatory elements that are active in the wing disc. A 4.3-kb region from position –4428 to –61 bp upstream of the hh transcription initiation site, which we named ABC (Fig. 1B), drove lacZ reporter expression in a pattern that reproduced the hh expression pattern in the wing imaginal disc and in the embryonic ectoderm [Fig. 1C; data not shown; number of independent lines analysed (n)=16]. We observed that lacZ expression was more robust in the distal portion of the wing disc, the presumptive wing pouch (encircled region in Fig. 1C). We next tested whether the ABC region integrates the genetic and epigenetic mechanisms involved in the regulation of endogenous hh (Fig. 1A) (Bejarano and Milan, 2009). En and Invected are expressed in the P compartment where they are required to repress Ci expression (Eaton and Kornberg, 1990) and relieve Ci-mediated repression of endogenous hh (Bejarano and Milan, 2009). Consistent with that, expression of a truncated form of Ci that behaves as a constitutive transcriptional repressor (CiCell) (Méthot and Basler, 1999) reduced ABC expression in P cells (compare Fig. 1D and 1E). PcG genes are involved in repression of hh in the A compartment (Randsholt et al., 2000) and, as shown in Fig. 1F, clones of cells located in the A compartment and mutant for the PcG gene Suppressor 2 of zeste [Su(z)2] showed ectopic expression of hh. In these clones, expression of the ABC reporter was also induced.

Fig. 1. ABC recapitulates the expression pattern of hh in the wing disc and behaves as a PcG response element (PRE) in Drosophila wing discs and adult eyes.

(A) Top: Wing discs carrying the hh-lacZ enhancer trap labelled to visualize β-gal protein expression. Bottom: Schematic of the genetic and epigenetic mechanisms involved in the regulation of hh expression in the wing primordium. White, anterior compartment; red, posterior compartment. (B) Genomic organization of the hh locus where the 4.3-kb-long genomic region (ABC) upstream of the hh transcription start site is depicted in red. (C-G) Wing discs carrying the ABC-lacZ reporter construct (C-E,G) or the hh-lacZ enhancer trap (F) and labelled to visualize in green Ci (C,D) or GFP (E,F,G) and in red or white β-gal protein expression. In E, C

β-gal and GFP were expressed under the control of the ap-gal4 driver and the anterior-posterior (AP) compartment boundary is depicted by a dashed line. In E and G, clones of cells mutant for Df(2R)7 and the PcG gene Su(z)2, and labelled by the absence of GFP are shown. In C, the yellow arrow points to a group of cells not expressing lacZ in the posterior compartment and the wing pouch is encircled. (H-O) Transgenic reporter assays for PRE function and expression in the wing disc. Wing discs (H-K) or adult eyes (L-O) of transgenic flies carrying a lacZ and a mini-white reporter construct containing the ABC region of hh [ABC-lacZ or ABC-Pviw] show expression of lacZ in the posterior (P) compartment of the wing (H-K), variegation (H-L, in adult eyes), pair-wise silencing (I,M, in adult eyes), loss of silencing in the PcG mutant background Su(z)2+/+ (J,N) and reduced activation in the trxG mutant background brm(trx+)(K,O). Wing discs shown in H-K were labelled to visualize Ptc (green) and β-gal (red and white) protein expression. Yellow arrows in H,J point to groups of cells not expressing lacZ in the posterior compartment of wing discs. A, anterior; P, posterior.
was less variegated in wing discs and adult eyes of **brahma** and reduction in the doses of the PcG gene animals. Both types of variegation were also sensitive to a transgene was usually more variegated than in heterozygous expression or eye colour of individuals homozygous for the chromosome (Vista Genome Browser, data not shown), we subdivided the 4.3-kb region upstream of the **hh** transcription start site in three fragments (A, B and C) (Fig. 1B) and generated reporter constructs carrying them. The A fragment drove some expression of **lacZ** in a patchy manner in wing discs, thereby indicating that it contains some wing enhancers (Fig. 2A, n=8). Most interestingly, A drove expression of **lacZ** and mini-white in a variegated manner in wing discs and adult eyes (Fig. 2A,E; see Table S1 in the supplementary material). The ABC cis-regulatory element contains the previously described **hh**-ME (Maurange and Paro, 2002). Consistent with this, **ABC-lacZ** expression in the P compartment was frequently repressed or variegated, with some groups of cells losing **lacZ** expression (Fig. 1C,H, yellow arrows). The mini-white reporter gene used to identify transgenic animals also showed a large degree of variegation in adult eyes (Fig. 1L; see Table S1 in the supplementary material). Thus, **ABC-lacZ** reporter in wing cells (Fig. 1, yellow arrows) and of mini-white in adult eyes (Fig. 1M; see Table S1 in the supplementary material). Thus, **ABC-lacZ** expression or eye colour of individuals homozygous for the transgene was usually more variegated than in heterozygous animals. Both types of variegation were also sensitive to a reduction in the doses of the PcG gene **Su(z)2** and the TrxG genes **brahma** and **trithorax**. Expression of the corresponding reporters was less variegated in wing discs and adult eyes of **Su(z)2/+** animals (Fig. 1J,N), and was largely reduced in **brahma trithorax** double heterozygous animals (Fig. 1K,O). Together, these results indicate that the ABC region integrates the genetic and epigenetic mechanisms involved in the regulation of **hh**. Other cis-regulatory regions might also be involved in the control of endogenous expression of this gene, as the ABC fragment drove variegated expression of **lacZ** in the P compartment and this expression was sensitive to changes in the doses of PcG and TrxG genes whereas a **hh-lacZ** enhancer trap drove robust expression in all P cells and this expression was not sensitive to changes in the doses of PcG and TrxG genes (data not shown).

**Distinct functional modules within the ABC cis-regulatory element**

On the basis of sequence conservation with other *Drosophila* species (Vista Genome Browser, data not shown), we subdivided the 4.3-kb region upstream of the **hh** transcription start site in three fragments (A, B and C) (Fig. 1B) and generated reporter constructs carrying them. The A fragment drove some expression of **lacZ** in a patchy manner in wing discs, thereby indicating that it contains some wing enhancers (Fig. 2A, n=8). Most interestingly, A drove expression of **lacZ** and mini-white in a variegated manner in wing discs and adult eyes (Fig. 2A,E; see Table S1 in the supplementary material). The ABC cis-regulatory element contains the previously described **hh**-ME (Maurange and Paro, 2002). Consistent with this, **ABC-lacZ** expression in the P compartment was frequently repressed or variegated, with some groups of cells losing **lacZ** expression (Fig. 1C,H, yellow arrows). The mini-white reporter gene used to identify transgenic animals also showed a large degree of variegation in adult eyes (Fig. 1L; see Table S1 in the supplementary material). Thus, **ABC-lacZ** reporter in wing cells (Fig. 1, yellow arrows) and of mini-white in adult eyes (Fig. 1M; see Table S1 in the supplementary material). Thus, **ABC-lacZ** expression or eye colour of individuals homozygous for the transgene was usually more variegated than in heterozygous animals. Both types of variegation were also sensitive to a reduction in the doses of the PcG gene **Su(z)2** and the TrxG genes **brahma** and **trithorax**. Expression of the corresponding reporters was less variegated in wing discs and adult eyes of **Su(z)2/+** animals (Fig. 1J,N), and was largely reduced in **brahma trithorax** double heterozygous animals (Fig. 1K,O). Together, these results indicate that the ABC region integrates the genetic and epigenetic mechanisms involved in the regulation of **hh**. Other cis-regulatory regions might also be involved in the control of endogenous expression of this gene, as the ABC fragment drove variegated expression of **lacZ** in the P compartment and this expression was sensitive to changes in the doses of PcG and TrxG genes whereas a **hh-lacZ** enhancer trap drove robust expression in all P cells and this expression was not sensitive to changes in the doses of PcG and TrxG genes (data not shown).
Expression of both reporters showed pairing-sensitive silencing (Fig. 2B,F; see Table S1 in the supplementary material) and was modulated by changes in the doses of PcG (Fig. 2C,G) and TrxG genes (Fig. 2D,H). These results indicate that the 2.1-kb long A fragment behaves as a PRE. Consistent with these data, chromatin immunoprecipitation (ChIP) on chip assays to map the chromosomal distribution of Gaf (Trl–FlyBase), Pho, Phol (Phol–FlyBase) and Dsp1, four DNA-binding proteins thought to be involved in PcG recruitment, identified this region as a potential PRE (Schuettengruber et al., 2009).

The reporter constructs carrying the B or C fragments did not show variegation in adult eyes (see Table S1 in the supplementary material) and expression of mini-white was unaffected by changes in the doses of PcG or TrxG genes (data not shown). In wing discs, the B fragment did not drive expression of lacZ (Fig. 2I, n=8), whereas the C fragment drove expression in a posterior wedge straddling the DV compartment boundary (Fig. 2J,K, n=5). Some expression in anterior cells abutting the AP and DV compartment boundaries was observed in C-lacZ wing discs (Fig. 2J,K, yellow arrows). As the Notch signalling pathway is activated at the DV boundary (Irvine and Vogt, 1997), we examined whether C-lacZ expression depends on the activity of Notch. For this purpose, we analysed the effects on reporter expression after blocking or activating this pathway. Expression of a dsRNA form of Notch (N\textsubscript{dsRNA}) in dorsal cells induced a cell-autonomous loss of C-lacZ expression (Fig. 2M), and expression of a dominant active form of Notch (N\textsubscript{intr}) in the same cells led to an expansion of C-lacZ expression throughout the D compartment (Fig. 2O). We analysed next the role of Ci in regulating the expression of C-lacZ. A repressor form of Ci (Ci\textsuperscript{cell}) induced a cell-autonomous loss of C-lacZ expression (Fig. 2L,N). Ectopic expression of Engrailed in anterior cells is known to repress ci expression (Eaton and...
Kornberg, 1990) and we showed that it induced the expression of C-lacZ along the anterior DV boundary (Fig. 3G). Together, these results indicate that the C fragment integrates the activities of the Notch pathway and the CiP. Interestingly, canonical binding sites for Ci and the transcriptional factor Su(H) are found in this fragment (Fig. 2P) and chromatin-binding assays identified this region as a binding domain for CiP, Su(H) and Notch (Biehs et al., 2010) (S. Bray, personal communication).

**Enhancer-PRE communication mediates epigenetic inheritance of gene expression**

The results above indicate that C contains the enhancers that drive expression to a posterior wedge straddling the DV compartment boundary whereas A behaves as a PRE. This PRE has been reported to be able to maintain Gal4-driven lacZ reporter gene expression through several rounds of mitosis (Bejarano and Milan, 2009; Maurange and Paro, 2002). It is known that most wing pouch cells are progeny of the cells determined at the DV boundary at early larval stages (Klein, 2001). Thus, we hypothesized that transcription of hh is activated, through the C enhancer, in boundary cells during early larval development. The active transcriptional state should then be inherited, by the activity of the hh-PRE, to daughter cells after mitosis. This should result in expression of the gene in all wing pouch cells. We undertook the following experimental approaches to evaluate this hypothesis.

First, we examined the expression induced by C during wing development. In early wing primordia, C drove lacZ expression to a posterior wedge straddling the DV boundary; this posterior wedge occupies a large fraction of the wing pouch and this fraction was reduced as the wing disc grew in size (Fig. 3A). This comparison suggests that cells lose lacZ expression as they are displaced out of the C domain by growth of the disc and that most of the wing pouch cells in the posterior compartment are born in the C domain. To verify that this is indeed the case, we lineage-tagged cells born in the C-expressing region using C-Gal4 to direct expression of FLP recombinase. In larvae carrying C-Gal4, UAS-FLP and act5c>stoP>lacZ, FLP recombinase is expressed in cells expressing C-Gal4 and mediates excision of the flip-out ‘stop’ cassette from the inactive reporter construct to generate an active act5c>lacZ transgene (Struhl and Basler, 1993). After excision of the cassette, reporter gene expression is regulated by the actin promoter and is clonally inherited in all the progeny of C-Gal4-expressing cells in which the recombination event took place. In C-Gal4, UAS-FLP, act5c>stoP>lacZ wing discs, expression of lacZ was expanded throughout most of the posterior wing pouch (Fig. 3B, compare with the expression of the C-enhancer in mature wing primordia labelled by the expression GFP). These results indicate that most of the wing pouch cells in the posterior compartment are born in the C domain.

We next analysed the capacity of A and iab-7 to mediate epigenetic inheritance of C-induced expression and to expand, by growth, the expression domain of C throughout the posterior wing pouch. The combination of A and C was able to expand the expression domain of C throughout the most distal part of the wing disc (compare Fig. 3C and 3D, n=4), and in the presence of the B fragment this expansion took place only in P cells (compare Fig. 3E and 3F, see below, n=16). Interestingly, iab-7, a well characterized ME of Abdominal B (Abd-B) (Hagstrom et al., 1997; Mishra et al., 2001), in combination with B and C (in iab-7-BC-lacZ wing discs), also expanded the expression domain throughout the most distal part of the P compartment (Fig. 3K, n=3) and this expansion depended on the doses of PcG and TrxG genes (Fig. 3L,M). We also tested the capacity of A and iab-7 to mediate epigenetic inheritance of C-induced expression upon ectopic expression of En in the anterior compartment. Ectopic expression of En along the DV compartment boundary (with the vg-BE-gal4 driver) induced expression of C-lacZ or BC-lacZ only in those anterior cells with high levels of En protein (Fig. 3G,I). By contrast, this expression was maintained throughout the wing pouch in the absence of detectable levels of En when the constructs also carried the A fragment or the iab-7 PRE (e.g. AC-lacZ, ABC-lacZ or iab-7-BC-lacZ, Fig. 3H,J,N). Note, as shown in Fig. 6C, that most of the wing pouch cells were born in the vg-BE domain. Together, these results indicate that communication between a PRE (A) and the enhancers located in the C module drive sustained expression throughout the posterior wing pouch.

**Enhancer-PRE communication mediates repression of gene expression**

Whereas the ABC domain of hh drove restricted and sustained expression of lacZ in the posterior compartment of the developing wing (Fig. 1), the absence of the B fragment (in AC-lacZ reporter constructs) failed to repress lacZ expression in anterior cells close to the AP boundary (Fig. 4A, white arrow). In order to address the role of B in mediating repression in the anterior compartment, we combined it with other modules and analysed the resulting expression patterns. B in combination with A was indistinguishable

![Fig. 4. Repression in the anterior compartment. (A-H) Wing discs carrying the following reporter constructs: AC-lacZ (A), BC-lacZ (B), AB-lacZ (C-F), AxC-lacZ (G), ACB-lacZ (H) were labelled to visualize in green Patched (Ptc) and in red or white β-gal protein expression. AB showed variagated lacZ expression (C), pairing-sensitive silencing (D), loss of silencing in a PcG mutant background (E) and reduced activation in a TrxG mutant background (F). In A and B, the anterior (A) and posterior (P) compartments are labelled and white arrows point to the expression of β-gal in the anterior compartment. In G and H, the ratio of wing discs with or without ectopic expression of lacZ in the anterior compartment (white arrows) is shown.](image)
from A alone (compare Fig. 2A-D and Fig. 4C-F) and the resulting construct, AB, drove expression of lacZ and mini-white in a variegated manner in wing discs and adult eyes (Fig. 4C, n=2; data not shown; see Table S1 in the supplementary material). Reporter expression driven by AB showed pairing-sensitive silencing in wing and eye tissues (Fig. 4D and data not shown; see Table S1 in the supplementary material) and this expression was modulated by changes in the doses of PcG and TrxG genes (Fig. 4E,F; data not shown). Thus, the features of A as a PRE were not visibly affected by the presence of B. Similarly, B in combination with C was indistinguishable from C alone (compare Fig. 4B and Fig. 2J) and the resulting construct, BC, drove expression in a posterior wedge straddling the DV compartment boundary (n=5). Some anterior cells expressed lacZ, as observed with the C alone (Fig. 4B, white arrow).

All together, these results indicate that B participates in mediating repression of lacZ in anterior cells receiving the Hh signal only in the presence of both A and C fragments (Fig. 1C).

Interestingly, an ACB fragment, where the order of the A, B and C fragments was altered, drove restricted and sustained expression of lacZ in the posterior compartment (Fig. 4H, n=20/24), suggesting that B contains those enhancers that respond, in the presence of A and C, to the mechanisms involved in the repression of anterior hh expression. However, a chimeric A-C fragment, in which the B element was substituted by an heterologous DNA fragment (the λ phage) of the same size (600 bp), also gave similar results in terms of restricted and sustained expression of lacZ in all P cells (Fig. 4G, n=15/24), suggesting that B also serves as a scaffold in mediating communication between the A and C modules. Thus, B plays a dual and redundant role in the repression of hh expression in A cells. Interestingly, we noticed that some wing discs carrying the A-C-lacZ or A-CAB-lacZ reporters showed some lacZ expression in the anterior compartment (Fig. 4G, n=9/24, and Fig. 4H, n=4/24, white arrows), suggesting that the dual role of the B fragment confers robustness to the repression of gene expression in anterior cells.

It has been reported that the hh gain-of-function allele Moonrat (hhMrt) results in de-repression of hh in anterior cells located at the DV boundary (Fig. 5E,F) and causes duplication of both A and C fragments (Fig. 5A,B) (de Celis and Ruiz-Gomez, 1995; Felsenfeld and Kennison, 1995). The adult wing phenotype is enhanced when halving the doses of PcG and TrxG genes (Fig. 4E,F; data not shown) and this expression was modulated by changes in the doses of PcG and TrxG genes (Fig. 4E,F; data not shown).

Enhancer-PRE communication mediates epigenetic inheritance of gene expression: is this a general mechanism?

Our results indicate that communication between a PRE (A) and the enhancers located in the C fragment that respond to CiP and Notch drive sustained expression of hh throughout the posterior wing pouch. We then questioned how generally this mechanism is used in development. vestigial (vg), a gene required for growth and cell survival of wing cells, is expressed in the wing pouch in a graded manner at both sides of the DV boundary (Fig. 6A) (Kim et al., 1996). vg expression is regulated by the combined activities of the Notch pathway and the Wingless signalling molecule. The so-called boundary enhancer (vg- BE, located in the second intron of vg, Fig. 6K) drives expression to the DV boundary (Fig. 6B, n=8) and responds to the activity of Notch (Kim et al., 1996), whereas the quadrant enhancer (vg- QE, located in the fourth intron, Fig. 6K) drives expression in non-boundary cells and responds to the activity of Wingless, a signalling molecule expressed at the DV boundary.
Intriguingly, vg can be expressed in the wing pouch in the absence of Wingless protein at the DV boundary or in cells lacking the Wingless receptor (Piddini and Vincent, 2009). These surprising results suggest that vg expression in the wing pouch is not only regulated by Wingless but also by other redundant mechanisms. Interestingly, genome wide bioinformatic prediction of PRE/TREs regulated by Wingless but also by other redundant mechanisms showed pairing-sensitive silencing (Fig. 6F; see Table S1 in the supplementary material), reported (Lee et al., 2005), variegation of the lacZ BE throughout the wing disc. vg-PRE did not drive expression of expression and to expand, by growth, the expression domain of vg-BE to mediate epigenetic inheritance of vg-BE-induced silencing of a locus that was confirmed by ChIP as a PcG binding site in S2 cells (Ringrose et al., 2003). In transgenic assays, this PRE caused variegation and pairing sensitive silencing of a mini-white gene in adult eyes (Fig. 6E,F; see Table S1 in the supplementary material) (see also Lee et al., 2005). We then addressed whether communication between this PRE and the vg-BE contributes to expansion of the expression domain of vg at both sides of the DV boundary in a similar manner to hh. Again, we undertook the following experimental approaches to evaluate this hypothesis. First, we lineage-tagged cells born in the vg-BE-expressing region using vg-BE-Gal4 to direct expression of FLP in larvae carrying the act5c>sto>P(w+) cassette (Struhl and Basler, 1993). In these wing discs, expression of lacZ was expanded throughout most of the wing disc (Fig. 6C) (see also Vegh and Basler, 2003), indicating that most of the wing disc cells are born in the vg-BE domain. Next we analysed the capacity of the potential vg-PRE to mediate epigenetic inheritance of vg-BE-induced expression and to expand, by growth, the expression domain of vg-BE throughout the wing disc. vg-PRE did not drive expression of lacZ in wing disc cells (Fig. 6D, n=10) and induced, as previously reported (Lee et al., 2005), variegation of the mini-white gene in adult eyes (Fig. 6E; see Table S1 in the supplementary material), showed pairing-sensitive silencing (Fig. 6F; see Table S1 in the supplementary material) and was modulated by changes in the doses of PcG (Fig. 6G) and TrxG genes (Fig. 6H). Interestingly, the combination in cis of vg-PRE with vg-BE drove expression of lacZ reporter not only in DV boundary cells but also at both sides of the DV boundary in a variegated manner (Fig. 6I, n=4), indicating that the presence of the vg-PRE was able to induce expansion of the expression domain of vg-BE at both sides of the DV boundary. A second PRE located in the vg promoter region was identified by ChIP on chip assays as an enrichment site in PcG proteins (Schuettengruber et al., 2007). However, this PRE was not able to induce expansion of the expression domain of vg-BE at both sides of the DV boundary (data not shown).

Finally, we analysed whether enhancers and PREs from different genes were interchangeable and worked in a modular manner. For this purpose, we combined in cis vg-PRE with the hh-PRE (the A module) and analysed the resulting expression pattern. Interestingly, the expression domain of vg-BE was expanded throughout the wing pouch in the presence of the A module of hh (Fig. 6J, n=5). This observation together with the fact that the PRE of Abd-B (iab-7) was able to expand the expression domain of the C enhancer of hh when located in cis (Fig. 3K,L) strongly suggest that enhancers and PREs are interchangeable modules whose communication contributes to the expansion of gene expression in growing tissues.

**DISCUSSION**

*Bithorax (BX-C) and Antennapedia* homeotic gene complexes determine the segmental identity of the fly along the anterior-posterior axis and functional analysis of the >300-kb cis-regulatory
region of the BX-C complex has been very illustrative with regard to understanding the modular role of enhancers and MEs in the initiation and maintenance of expression of the three BX-C homeotic genes Ubx, abd-A and Abd-B along the anterior-posterior axis of the fly embryo (for a review, see Maeda and Karch, 2006). Here, we provide evidence that communication between enhancers and MEs are also involved in the initiation and maintenance of gene expression within the growing primordium of the fly wing. Functional analysis of the 4.3-kb cis-regulatory region upstream of hh has been very illustrative in this regard (Fig. 7). We have identified a cis-regulatory element (termed C) that contains the enhancers that respond to the activity of the repressor form of Ci (CiRPP) (see also Biehs et al., 2010) and the Su(H) transcription factors, and initiates gene expression, from the early wing primordium stage, in a posterior wedge that corresponds to the DV compartment boundary (Fig. 7A). Transcriptional activation is maintained by the activity of a previously identified ME in cis and is expanded, by tissue growth, throughout the posterior compartment (Fig. 7B). This expansion depends on the activity of PcG and TrxG genes, as changes in the doses of these genes modulate the capacity of the ME to maintain the expression in posterior cells. We underscored a dual role of a fragment of 600 bp located between the C enhancer and the ME in mediating repression of gene expression in anterior cells.

Interestingly, repression and expansion of gene expression can also be mediated by the presence of the ME of Abd-B in cis with the C element. This observation thus reinforces the proposal that enhancer-ME communication mediates repression and epigenetic inheritance of gene expression (Muller and Kassis, 2006) and that MEs are interchangeable modules (Americo et al., 2002; Chiang et al., 1995; Kozma et al., 2008). It is interesting to note in this context that enhancer-PRE communication appears to contribute to the expansion of the expression domains of other developmental genes, such as vg, and that these enhancers also behave as interchangeable modules. Thus, communication between the vg-BE enhancer, which responds to the activity of Su(H) and is expressed in cells at the DV boundary, and a vg-PRE is able to expand, to some extent, the expression to wing pouch cells. This expansion can also be mediated by the presence of the hh-ME when in cis with vg-BE. Hence, on the basis of our findings we propose that enhancer-ME communication is a general mechanism at work in highly proliferative tissues that contributes to the expansion of the expression domains of developmental genes.

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References


Fig. 7. Enhancer-PRE communication contributes to hh expression in the Drosophila wing. (A) The combined activities of C in anterior cells and Notch in cells along the dorsal-ventral (DV) compartment boundary act through the hh-C Enhancer and result in the initial induction of gene expression to a posterior wedge straddling the DV boundary. Note expression in anterior cells abutting the anterior-posterior (AP) compartment boundary (AP/A). Communication between the hh-C Enhancer and the hh-A maintenance element contributes to the expansion, through growth, of gene expression in the posterior compartment and to the repression of gene expression in the anterior compartment. The hh-B element plays a dual role in the repression of gene expression in anterior cells. As a consequence, expression of hh (red) is restricted to the posterior compartment. (B) Genomic organization of the hh locus in which the ABC upstream region of the hh transcription start site is depicted in red and the different molecules acting on the hh-A maintenance element (PcG and TrxG protein) and the hh-C Enhancer (Ci and Notch) are shown.


