Opposing inputs by Hedgehog and Brinker define a stripe of *hairy* expression in the *Drosophila* leg imaginal disc

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Summary

The sensory organs of the *Drosophila* adult leg provide a simple model system with which to investigate pattern-forming mechanisms. In the leg, a group of small mechanosensory bristles is organized into a series of longitudinal rows, a pattern that depends on periodic expression of the *hairy* gene (\(h\)) and the proneural genes *achaete* (*ac*) and *scute* (*sc*). Expression of *ac* in longitudinal stripes in prepupal leg discs defines the positions of the mechanosensory bristle rows. The *ac/sc* expression domains are delimited by the *Hairy* repressor, which is itself periodically expressed. In order to gain insight into the molecular mechanisms involved in leg sensory organ patterning, we have analyzed a Hedgehog (\(Hh\))- and Decapentaplegic (\(Dpp\))-responsive enhancer of the *h* gene, which directs expression of *h* in a narrow stripe in the dorsal leg imaginal disc (the *D-h* stripe). Our studies suggest that the domain of *D-h* expression is defined by the overlap of \(Hh\) and high-level \(Dpp\) signaling. We find that the *D-h* enhancer consists of a \(Hh\)-responsive activation element (\(HHRE\)) and a repression element (\(REPE\)), which responds to the transcriptional repressor Brinker (\(Brk\)). The \(HHRE\) directs expression of *h* in a broad stripe along the anteroposterior (AP) compartment boundary. \(HHRE\)-directed expression is refined along the AP and dorsoventral axes by \(Brk1\), acting through the \(REPE\). In *D-h*-expressing cells, \(Dpp\) signaling is required to block \(Brk\)-mediated repression. This study elucidates a molecular mechanism for integration of the \(Hh\) and \(Dpp\) signals, and identifies a novel function for \(Brk\) as a repressor of \(Hh\)-target genes.

Key words: *hairy*, *hedgehog*, *decapentaplegic*, *brinker*, Mechanosensory organs, Leg imaginal disc development, *Drosophila*

Introduction

Pattern formation in vertebrates and invertebrates is controlled by several conserved signaling molecules that cooperate to specify particular cell fates at different threshold concentrations (Gurdon and Bourillot, 2001; Tabata, 2001). Crucial to this process are the cellular responses that give rise to the exquisitely patterned body plans of multicellular organisms. One important response to signals is the establishment of position-specific expression of genes that function at a local level to specify cell fates. The *Drosophila melanogaster* adult leg provides a simple model system with which to investigate this process. On the surface of the *Drosophila* leg, a group of small mechanosensory bristles, called microchaetae, are organized in a series of longitudinal rows around the leg circumference. The orderly arrangement of the leg microchaetae depends largely on the periodic expression of just a few genes, including the antineural gene, *hairy* (*h*), and the redundant proneural genes, *achaete* (*ac*) and *scute* (*sc*) (Orenic et al., 1993). Hence, mechanistic insight into the periodic patterning of leg microchaetae can be gained by investigating the regulation of *h* and *ac/sc* expression.

Extensive studies on the development of sensory structures in the *Drosophila* mesothorax and other tissues have shown that the redundant proneural genes, *ac* and *sc*, function at a local level to confer neural competence to cells destined to become sensory organs (SOs) (Calleja et al., 2002; Modolell, 1997). The bHLH transcription factors, Ac and Sc, are expressed in proneural clusters, groups of cells that roughly define the positions of future sensory structures in the adult (Cubas et al., 1991; Romani et al., 1989; Skeath and Carroll, 1991). Then, through local regulatory events controlled by the neurogenic genes, a cell(s) is selected from each proneural cluster to become a sensory organ precursor, which undergoes a few differential cell divisions (Calleja et al., 2002; Modolell, 1997). The resulting cells give rise to the components of the SO. In prepupal legs, expression of *ac* and *sc* in a series of longitudinal proneural stripes around the leg circumference defines the primordia of the mechanosensory microchaetae. Expression of *ac* in the leg is regulated by *h* (Orenic et al., 1993), which is itself periodically expressed in two pairs of longitudinal stripes, one pair that traverses the dorsoventral (DV) axis (DV-*h*) and another pair that runs along the anteroposterior (AP) axis (AP-*h*) (Carroll and Whyte, 1989; Hays et al., 1999). On either side of each *h* domain, a stripe of *ac* expression demarcates the position of each leg microchaete bristle row (Orenic et al., 1993). *h* encodes a bHLH transcription factor that regulates the expression of *ac*. This study elucidates a molecular mechanism for integration of the *Hh* and *Dpp* signals, and identifies a novel function for Brk as a repressor of *Hh*-target genes.
transcription factor (Rushlow et al., 1989) and is a direct repressor of ac expression (Ohsako et al., 1994; Van Doren et al., 1994). In the absence of h function, ac expression expands into the regions normally occupied by Hairy, broadening the microchaete proneural fields and resulting in disorganized bristle rows in the adult. Therefore, precise position-specific expression of h in leg discs is crucial for generation of the adult leg bristle row pattern.

We have investigated the regulation of two h stripes, the DV-h stripes, and have found that they are established in response to the signaling molecules that globally pattern leg imaginal discs (Hays et al., 1999). Expression of the DV-h stripes is controlled by a pair of modular enhancers that direct expression of the dorsal (D-h) and ventral (V-h) stripes, respectively (Fig. 1). Here, we focus on the function of the D-h stripe enhancer, which directs expression of h in a narrow dorsal domain positioned a few cells anterior to the AP-compartment boundary and integrates input from the Hedgehog (Hh) and Decapentaplegic (Dpp) signaling pathways.

In leg imaginal discs, Hh secreted by posterior compartment cells signals to anterior cells to activate expression of dpp in a primarily dorsal stripe near the AP boundary (Diaz-Benjumea et al., 1994). Hh activates gene expression through its transcriptional mediator Cubitus interruptus (Ci), a zinc-finger transcription factor (Alexandre et al., 1996; Dominguez et al., 1999; Methot and Basler, 1996; Methot and Basler, 2000; Wang and Holmgren, 1999; Wang and Holmgren, 2000).

Dpp acts as a long-range morphogen and regulates gene expression in imaginal discs in a concentration-dependent manner (Lecuit et al., 1996; Nellen et al., 1996). In response to Dpp signaling, an activated form of the Smad transcription factor, Mothers against dpp (Mad), is generated. Mad then binds to a related protein, Medea (Med), and this complex translocates to the nucleus to transcriptionally regulate expression of Dpp target genes (Raftery and Sutherland, 1999; Wang and Holmgren, 1999; Methot and Basler, 1996; Methot and Basler, 2000; Wang and Holmgren, 1999; Wang and Holmgren, 2000).

Our genetic studies suggest that Hh and Dpp signaling are both required to activate D-h expression (Hays et al., 1999). Based on these genetic observations, we proposed that Ci and Mad/Med act synergistically to activate h expression in the dorsal region of the leg disc. To test this model and to investigate the molecular mechanisms underlying integration of the Hh and Dpp signals, we have undertaken a molecular analysis of the D-h enhancer. We show that the D-h enhancer consists of at least two distinct sub-elements: a Hh-responsive element (HHRE), which directs expression in a broad AP boundary stripe, and a repression element (REPE), which refines HHRE-directed expression along the AP and DV axes. We find that Brk acts through the REPE to repress HHRE-directed expression, while Dpp functions to block Brk-mediated repression in a narrow dorsal stripe. These observations suggest a novel role for Brk in repression of a Hh-target gene and elucidate a mechanism for the integration of the Hh and Dpp signals. Furthermore, this study establishes a correlation between enhancer function and the formation of specific morphological elements, the dorsal microchaete bristle rows of the adult leg.

Materials and methods
Fly strains and genetics
The following fly strains were used in this study: smo1HG26 FRT40ACyo (Blair and Ralston, 1997), dpp6CyO, dpp412CyO (Spencer et al., 1982), w; wGsp-1CyO; dpp6Gal4/TM6B, Tbl (Staehling-Hampton et al., 1994), y w brk3 f26c FRT18A/FM7 (Minami et al., 1999), UAS-brk, brk-lacZ (Jazwinska et al., 1999), UAS-tkQ253D (Nellen et al., 1996) and D-h-lacZ (Hays et al., 1999). smo1HG26 is a strong hypomorphic mutation (van den Heuvel and Ingham, 1996). The dpp6CyO, dpp412CyO, w; wGsp-1CyO; dpp6Gal4/TM6B, Tbl, dpp-lacZ, UAS-GFP and FRT strains were obtained from the Bloomington Stock Center. The following transgenic lines were generated for this study (see below for details): D-h-GFP, D-h-Ci-lacZ, D-h-MM-GFP, D-h-MM-GFP, D-h-C-GFP, D-h-CB-GFP, D-h-MCM-GFP, D-h-M2-GFP, D-h-BM2-GFP, HHRE-lacZ, HHRE-GFP, HHRE-Ci1-lacZ, HHRE-Ci2 and HHRE-Ci1+2-lacZ.

smo and brk mutant clones were made in larvae of the genotypes: y hs-flp/HHRE-GFP; smo1HG26 FRT40A hs-piMyc36F FRT40A or brk FRT18A hs-piMyc10D FRT18A; D-h-GFP/+; hs-flp/+ . Clones were generated by heat shocking larvae (48-96 hours after egg laying, AEL) for 1 hour at 37°C. Prior to dissection, third instar larvae or prepupae were heat shocked for 1 hour at 37°C to induce pUbi expression and were then allowed to recover for 1 hour.

For analysis of transgene expression in leg discs with reduced dpp function, discs were dissected from larvae or prepupae of the following genotypes: (1) D-h-GFP; dpp6dpp412; (2) HHRE-GFP; dpp6dpp412; (3) brk-lacZ; dpp6dpp412. For ectopic expression studies, leg imaginal discs were dissected from larvae or prepupae of the following genotypes: (1) D-h-GFP; UAS-tkQ253D/+; dpp-Gal4/+, (2) D-h-GFP; UAS-brk/+; dpp-Gal4/+, (3) HHRE-GFP; UAS-brk/+; dpp-Gal4/+, (4) D-h-CB-GFP; UAS-brk/+; dpp-Gal4 and (5) D-h-C-GFP; UAS-brk/+. dpp-Gal4.

Immunohistochemistry and microscopy
For anti-Myc (Xu and Rubin, 1993), anti-β-galactosidase (Promega) and anti-Engrailed (Patel et al., 1989) staining, dissected imaginal discs or prepupal legs were treated as described (Carroll and Whyte, 1989). All images were collected on a Zeiss Axiovert 200 M equipped with a digital camera. Fluorescent images were collected as z-stacks and were subjected to 3D-deconvolution.
Generation of wild-type and mutant reporter constructs and transformation

To generate the lacZ-transgenes, the corresponding wild-type and mutant genomic DNA fragments were cloned in HSPCasper-lacZ (Nelson and Laughon, 1990). GFP transgenes were generated by cloning the corresponding wild-type and mutant genomic DNA into the pHStinger vector (Barolo et al., 2000). The pHStinger vector offers the advantage that the reporter gene is flanked by insulator sequences from the gypsy transposon. The insulator sequences reduce position effects and thus result in less variability in expression among different transgenic lines. Expression was assayed from at least three independent transgenic lines for each construct; similar expression levels were observed among all the lines. In addition, similar results were obtained with insertions in both vectors. The D-h transgenes were generated by cloning a 3.4 kb BamHI/EcoRI genomic fragment (see Fig. 1B and results) into the HSPCasper or pHStinger vectors. A 1 kb BamHI/HindIII subfragment (Fig. 1B) from the D-h enhancer was cloned into the corresponding vector to generate the HHRE (Hh response element, see results) transgene.

All the site mutations were generated using the QuickChange Site-Directed Mutagenesis Kit (Stratagene) or the USE Mutagenesis Kit (Pharmacia) and are as follows (altered bases are shown in lower case):

Ci binding site mutations in the HHRE: Wild type, GACCTCC-CA…………..GACCACCAT; Ci1, GACCTCCCA…………..GACCACCAT; and Ci1&2, GACCTCCCCA…………..GACCCCAT.

CMB site mutations in the D-h enhancer: Wild type, GCGAGC-GGCTCATC; CRE(C), GCGACGGGCGcCTC; Mad1/CRE/-Mad2(MCM), aattGGGCGcCTT; Mad1/Brinker/Mad2(MBM), aattGGGCGCATT; CRE/Brinker(CB), GCGAGGCGGAgCTG; Mad1/Mad2(MM), aattGGGCGCCTAa; Mad2(M2), GCGAGC-GGCTAa; Brinker/Mad2(BM2), GCGACAGGTCaag.

Prior to introducing the mutated fragments into flies, all the mutagenized regions were tested by gel mobility shift assays with Ci zinc-finger domain, Brinker and/or N-Mad proteins. Several versions of the MM mutant were tested; all affected Brk as well as Mad response element. (A) A third instar leg imaginal disc depicting hairy expression (green) and the compartment boundary. The hairy stripe is shown a more detailed map of the D-h enhancer. Below is shown a more detailed map of the D-h enhancer. Fragments tested in reporter constructs are shown beneath the detailed map: full-length D-h enhancer (light blue) and the Hh response element (HHRE, dark blue), which has two Ci binding sites and the repression element (REPE, red; see text and Fig. 3). The CMB site in the REPE is described in the text and in Fig. 8. (C) Sequence of two Ci-binding sites present in the HHRE. Underlined bases were altered in the C11, C12 or C11&2 double mutant (see Materials and methods). (D,E) lacZ expression in leg imaginal discs carrying: D-h-lacZ (D) and HHRE-lacZ (E). (F-G) lacZ expression directed by HHRE-lacZ site mutants: C11 mutant (F), C12 (G) and C11&2 (H).

Results

In a previous study, we reported that the D-h and V-h stripes are regulated by separate enhancers, which map between 32-38 kb 3' to the D-h transcription unit (Hayes et al., 1999). Expression of the DV-h stripes relative to their flanking ac stripes and the compartment boundary is depicted in Fig. 1A. Although the ac stripes are not expressed until 6 hours after puparium formation (APF), they are projected onto a third instar leg disc for the sake of clarity. The narrow D-h stripe is activated by a Hh-response element. (A) A third instar leg imaginal disc depicting expression of the DV-h stripes (blue) relative to ac expression (green) and the compartment boundary. The D-h and V-h stripes occupy distinct registers relative to the AP boundary. (B) (Top) map of the h locus showing the position of the D-h enhancer. Below is shown a more detailed map of the D-h enhancer. Fragments tested in reporter constructs are shown beneath the detailed map: full-length D-h enhancer (light blue) and the Hh response element (HHRE, dark blue), which has two Ci binding sites and the repression element (REPE, red; see text and Fig. 3).
positioned a few cells anterior to the boundary, allowing expression of two dorsal ac stripes in the anterior compartment. V-h, however, is expressed directly adjacent to the AP boundary so that there is only one ventral ac stripe in the anterior compartment. Expression of each h stripe in its proper register is essential for positioning of the ac stripes and consequently for sensory bristle patterning in the adult leg. We focus on the mechanisms that lead to expression of the D-h stripe in its precise register near the AP boundary.

A Hh responsive activation element is required for D-h enhancer activity

We previously showed that expression of the endogenous D-h stripe is dependent on Hh signaling (Hays et al., 1999). In order to identify sequences that mediate Hh responsiveness, we undertook a dissection of the D-h enhancer. The D-h enhancer maps to a 3.4 kb BamHI/EcoRI fragment located 32 kb 3’ to the h structural gene (Fig. 1B). In third instar leg imaginal discs, this fragment directs lacZ expression in a dorsally restricted AP boundary-adjacent stripe (Fig. 1D). Two subfragments of the D-h enhancer were tested for the ability to drive reporter gene expression in leg imaginal discs. A 3’ 2.4 kb HindIII/EcoRI subfragment of the D-h enhancer (REPE in Fig. 1B) directs no detectable reporter gene expression in leg imaginal discs (not shown). However, the complementary 5’ 1.0 kb BamHI/HindIII fragment of the D-h enhancer drives expression in a stripe that is not dorsally restricted but rather traverses the entire length of the DV axis (Fig. 1E), suggesting it responds to Hh signaling in both dorsal and ventral leg cells. To determine whether Hh signals through the BamHI/HindIII fragment of the D-h enhancer, expression from a BamHI/HindIII-GFP transgene was assayed in leg clones lacking function of Smoothened (Smo), a transmembrane protein required for transduction of the Hh signal (Alcedo et al., 1996; van den Heuvel and Ingham, 1996). Somatic clones lacking smo function were generated by FLP/FRT-mediated mitotic recombination (Xu and Rubin, 1993). We observed cell-autonomous loss of GFP expression in smo clones that overlapped the GFP stripe (Fig. 2A-C). These observations imply that Hh signals through the BamHI/HindIII fragment, and therefore, we refer to this region as the D-h-Hh response element (HHRE).

As the HHRE is Hh responsive, we searched the element for the consensus binding site of the Hh pathway transcriptional effector, Ci (Alexandre et al., 1996; Dominguez et al., 1996; Hepker et al., 1997; Von Ohlen et al., 1997). Two potential Ci-binding sites (Ci-1 and Ci-2) were found, each of which matches the consensus, TGGG(A/T)GGTC (Zarkower and Hodgkin, 1993), in a minimum of seven out of nine sites (Fig. 1C) and bind the Ci zinc-finger domain (CiZn) in an electrophoretic mobility shift assay (EMSAs) (see Fig. 3, lanes 1-7 show sequence-specific binding to the Ci-1 site). To determine whether the Ci-binding sites are required for HHRE-directed expression, point mutations were introduced into the Ci-1 and 2 sites (Fig. 1C). These mutations abolished Ci binding of the HHRE in vitro (Fig. 3, lane 7, Ci-1 mutant shown). Expression directed by the HHRE with a mutation in either the Ci-1 or Ci-2 sites is drastically compromised (Fig. 1F,G), and there is no detectable expression from an HHRE-lacZ transgene with both Ci sites mutated (Fig. 1H). Taken together, these studies indicate that D-h expression is activated primarily by the HHRE, through which Ci acts as an essential and direct transcriptional activator.

A D-h enhancer repression element restricts Hh-mediated activation of D-h

The D-h enhancer directs expression of a narrow GFP stripe, which coincides with endogenous D-h expression (Fig. 4A-C). HHRE-directed expression, however, is broader not only along the DV axis as observed in Fig. 1E (see also Fig. 5B) but also along the AP axis. Fig. 4D,E shows that the HHRE-GFP stripe extends a few cells anterior and posterior to the endogenous D-h stripe. These observations imply that sequences in the 3’ HindIII/EcoRI fragment of the D-h enhancer (Fig. 1B) function to repress ventral D-h expression and to restrict stripe width along the AP axis. We, therefore, refer to the HindIII/EcoRI fragment as the D-h-repression element (REPE).

It appears, then, that the spatial domain of D-h expression is established through broad activation by a Hh-responsive element and repression by an associated repressive element, the REPE. This refinement is presumably necessary to maintain the proper register of D-h expression relative to the compartment boundary, allowing proper positioning of the dorsal ac stripes and consequently the leg bristle rows. The register of D-h-GFP and HHRE-GFP stripes relative to engrailed (en) expression is shown in Fig. 4G-J. Note that the D-h-GFP stripe is positioned a few cells anterior to the compartment boundary, while HHRE-GFP expression extends up to the compartment boundary [the overlap between HHRE-GFP and en expression is due to low-level anterior compartment en expression which is observed beginning at the mid-third instar in wing discs (Blair, 1992) and after puparium formation in leg discs (Inaki et al., 2002)].

Dpp signaling is required to block repression of D-h expression

We have previously shown that endogenous D-h expression is compromised in somatic clones lacking function of Mad, the transcriptional effector of Dpp signaling, and that D-h-lacZ expression is severely decreased in leg imaginal discs with reduced dpp function (Hays et al., 1999). Furthermore, D-h-lacZ expression is ventrally expanded in wingless (wg) mutant

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Fig. 2. Hh signaling is required for function of the D-h activation element. Clones lacking smo function were examined in a prepupal leg, 3 hours APF. (A) HHRE-GFP expression (green). (B) myc expression (red). Homozygous smoG256 clones are identified by the lack of Myc expression. (C) Merge of images in A and B (several smo clones that overlap the HHRE-GFP stripe are outlined in white). Note the absence HHRE-GFP expression in smo clones.
Regulation of hairy leg expression

legs, which have strong ventral dpp expression (Brook and Cohen, 1996; Jiang and Struhl, 1996; Johnston and Schubiger, 1996; Morimura et al., 1996; Penton and Hoffmann, 1996; Theisen et al., 1996). These findings indicate a requirement for Dpp, in addition to Hh signal, for D-h expression. The most parsimonious model to explain how h integrates positive input from the Hh and Dpp signals, is that Mad acts synergistically with Ci through the D-h enhancer to activate D-h expression. However, we show here that Dpp is instead required to block REPE-mediated repression.

As shown above, the HHRE directs expression in a broad stripe that extends into the ventral leg (Fig. 1E, Fig. 5B), where there is little or no Dpp signaling, implying that HHRE-directed expression does not require Dpp function. To determine whether HHRE-directed expression is Dpp independent, D-h-GFP and HHRE-GFP expression were examined in dpp60(dpp112) leg imaginal discs, which have reduced dpp function. In these leg discs, D-h-GFP expression is severely compromised (compare Fig. 5A with 5F), while HHRE-GFP expression is unaffected (compare Fig. 5B with 5G; note that in both dpp mutant and wild-type leg discs, the HHRE-GFP stripe is 5-6 cells wide). This result suggests that Dpp signals through the REPE. As the REPE functions as a repressive element, Dpp probably functions to block the repressive effects of this element rather than to activate D-h expression.

In leg discs, dpp is expressed in a broad AP boundary adjacent stripe, which is stronger dorsally than it is ventrally.

Fig. 4. A D-h repression element attenuates activity of the activation element. (A-F) Comparison of endogenous Hairy [red in A,D (arrowhead indicates endogenous D-h stripe)] with D-h-GFP [green in B (arrowhead indicates D-h-GFP stripe)] and HHRE-GFP (green in E) expression in prepupal legs, 3 hours APF. (C) Merge of images in A and B. (F) Merge of images in D and E. en expression (red in G-J) relative to D-h-GFP (green in H) and HHRE-GFP (green in I) in prepupal legs 5 hours APF.
of coincides with a subset of cells expressing the highest levels of HHRE. In the wing and leg imaginal discs, repressor of Dpp target genes, Brk (Campbell and Tomlinson, 1999) directed expression. A potential candidate, the transcriptional repressor(s) that acts through the REPE to refine HHRE-enhancer (see dpp-lacZ expression, Fig. 5D). D-h-GFP expression coincides with a subset of cells expressing the highest levels of lacZ within the dpp stripe (Fig. 5C-E). Perhaps, then, high-level Dpp signaling functions, by overcoming repressive effects of the REPE, to define a narrow stripe of D-h expression within a broader region defined by Hh. A prediction stemming from this hypothesis is that elevation of Dpp signaling within the HHRE-response zone would expand D-h expression. To test this premise, Dpp signaling was elevated along the AP boundary by expression of a constitutively active form of the Dpp receptor Thickveins (TkVR) (Nellen et al., 1996). A dpp-Gal4 transgene (Staehling-Hampton et al., 1994), which drives Gal4 expression in a broad AP boundary stripe at high levels in the dorsal leg and more weakly in the ventral leg (Fig. 5H), was used to express UAS-tkVR. This results in broadening of the D-h-GFP stripe and partial expansion into the ventral disc (compare Fig. 5A with 5I). The insets in Fig. 5 show D-h- and HHRE-GFP expression in the basitarsal segment of leg discs dissected at 4 hours APF. By this time, the tarsal segments are further segmented and are clearly identifiable. In addition, HHRE-GFP expression in the basitarsal segment of leg discs (Fig. 6A). Furthermore, brk expression expands dorsally in dpp6/dpp12 legs (Fig. 6C), in which we showed that D-h expression is severely reduced (Fig. 5F).

To determine whether Brk functions as a repressor of D-h expression, we examined D-h-GFP expression in clones lacking brk function. Loss of brk function results in ectopic expression of D-h-GFP on either side of the D-h-GFP stripe. In Fig. 7A-C, ectopic expression is observed in clones anterior to the D-h-GFP stripe. However, the expansion is confined to a region two or three cells wide, directly juxtaposed to D-h expression, which presumably corresponds to the HHRE-responsive zone. In addition, we observe ectopic expression in ventral clones (Fig. 7D-F). We further observe that overexpression of brk along the AP boundary drastically reduces D-h-GFP expression but does not affect HHRE-GFP expression (Fig. 6D,E), indicating that Brk acts through the REPE to repress D-h expression. As D-h expression is activated primarily by the Hh-responsive HHRE, these observations identify Brk as repressor of Hh as well as Dpp target genes.

A Brinker responsive repression element refines D-h expression

Genetic data support a hypothesis in which Brk acts through the REPE of the D-h enhancer to modulate activity of the HHRE. If so, we might expect the REPE to contain one or more functional Brk-binding sites. Hence, we searched the REPE for the Brk consensus binding site, GCCG(C/T)(C/T) (Kirkpatrick et al., 2001; Sivasankaran et al., 2000; Zhang et al., 2001), and identified a potential Brk binding site which overlaps two sequences similar to a consensus binding sites for Mad, GCCGNGGC (Kim et al., 1997), and a sequence similar to a cAMP response element (CRE), TGACGTCA (Montminy et al., 1986). The sequence of overlapping CRE, Brk and Mad sites was designated the CMB element (see Fig. 1B, Fig. 8A).

To determine whether Brk might act through the CMB element, we first tested whether Brk binds the CMB site in vitro. EMSA analysis shows that Brk binds the CMB-Brk site in a sequence-specific manner (Fig. 3, lanes 8-14). In addition,
we observed sequence-specific binding of Mad to the two potential Mad-binding sites in the CMB (Fig. 3, lanes 15-22). Next, the function of the Brk binding site was tested by mutational analysis. It was not possible to alter the Brk site without compromising at least one other site so the following sites were mutated within the CMB: Mad2 (D-\(h\)-M2) and Brk/Mad2 (D-\(h\)-BM2) (Fig. 8A). Brk and Mad binding to all Mad and CRE mutant versions of the CMB was confirmed by EMSA (Brk binds the M2 mutant probe but not the BM2 mutant probe) (Fig. 3, lanes 25, 26). Although D-\(h\)-M2-GFP expression appears normal (Fig. 8B), D-\(h\)-BM2-GFP expression is broader along the AP and DV axes (Fig. 8C). These observations are consistent with the hypotheses that Brk acts through the CMB to repress D-\(h\) expression.

As shown above, mutation of the Mad2 site has no effect on D-\(h\)-GFP expression, suggesting that only the Mad1 site is required, that the Mad2 site is functionally redundant with the Mad1 site, or that neither site is required for D-\(h\) expression. To test whether the Mad-binding sites are involved in D-\(h\) expression, both CMB-Mad sites were mutated (D-\(h\)-MM) (Fig. 8A). Weak residual binding to a single Mad site is observed with the CMB-MM mutant probe (not shown; see Materials and methods) and Brk binding is also reduced (Fig. 3, lane 27). We observe that D-\(h\)-MM-GFP stripe extends into the ventral disc and is slightly expanded along the AP axis (Fig. 8D, note that the stripe is two or three cells wide in the pupal leg). A probable explanation for this result is that ectopic expression is caused by compromised Brk binding to the MM-mutant CMB. We also observe that a D-\(h\)-MM-GFP transgene directs expression of a stripe, which is broader along the AP and DV axes of the leg disc (Fig. 8E). The finding that the MBM mutation (which completely abrogates Brk binding) causes a more severe expansion phenotype than the MM mutation (which partially reduces Brk binding) is consistent with the hypothesis that the Brk binding site contributes to repression of D-\(h\) expression.

A Brinker response site in the D-\(h\) repression element is not sufficient to mediate repression

We also asked whether the CRE is required for proper D-\(h\) expression. To test function of this site, a mutation was introduced into the CRE (D-\(h\)-C), such that Brk and Mad binding were not compromised (Brk binding to the CRE mutant is shown in Fig. 3, lanes 24). Surprisingly, mutation of the CRE results in expression very similar to that of the HHRE-GFP transgene. The D-\(h\)-C stripe extends ventrally and is five or six cells wide, which suggests that the CRE is required for D-\(h\) repression (Fig. 8G). Very similar expression is observed with D-\(h\)-MB-GFP (not shown).
transgenes. Although, it appears that Brk binds the CMB to mediate repression of HHRE-directed expression, it is evident that an intact Brk binding site is not sufficient for D-h repression.

Because of the extensive overlap of Mad, CRE and Brk sites in the CMB, it was necessary to alter one base pair in the CRE when the BM2 and MBM mutations were generated. Although this mutation alters the site so that it more closely resembles a canonical CRE (Fig. 8A), it is possible that it disrupts function of the CMB-CRE. This raises the possibility that Brk acts indirectly through the CRE rather than by directly binding the Brk site. To test this hypothesis, we assayed the effect of Brk overexpression on D-h-C-GFP and D-h-CB-GFP expression. Brk overexpression only mildly affects D-h-CB-GFP (Fig. 6F) but severely reduces D-h-C-GFP (Fig. 6G) expression, suggesting that Brk can act directly through its binding site in the CMB.

**Discussion**

As a critical regulator of periodic ac/sc expression in the *Drosophila* leg disc, *h* performs a pivotal function in formation of adult leg morphology. We have previously shown that compromised function of the enhancers that control *h* expression in leg imaginal discs results in disruption of the adult leg microchaete pattern. Furthermore, in this and a previous study, we observed that the *h* leg stripe enhancers respond to distinct combinations and levels of the Hh, Dpp and Wg morphogens. Hence, *h* acts to link function of the morphogens that globally pattern leg imaginal discs to ac/sc, which act at a local level to define the primordia of the leg microchaete bristle rows. In order to gain insight into the molecular mechanisms underlying signal integration by the *h* gene, we undertook an analysis of the D-h enhancer. Dissection of the D-h enhancer led to the identification of activation and repression elements, which together establish a spatially defined stripe of *h* expression in the dorsal leg.

**Activation of D-h expression**

The D-h activation element, HHRE, has two consensus Ci-binding sites, which bind Ci in vitro, and are required for its activity. In addition, HHRE-GFP expression is abrogated in clones lacking function of *smo*, a transducer of the Hh signal (Alcedo et al., 1996; van den Heuvel and Ingham, 1996). These observations suggest that Ci acts directly through the HHRE to activate D-h expression. *h* is one of a number of genes, including *dpp*, *patched* (*ptc*), *knot* and *araucan/caup* (*ara/caup*), that have been identified as targets of Hh signaling in imaginal discs (Aza-Blanc and Kornberg, 1999; Vervoort, 2000). These genes are each expressed in a stripe along the AP compartment boundary, but curiously, stripe widths among the genes varies as does register relative to the AP boundary. This has been explained in terms of differential response of Hh-target genes to the repressor and activator forms of Ci (Ci-R and Ci-A, respectively) found in anterior compartment cells (Methot and Basler, 1999; Muller and Basler, 2000; Wang and Holmgren, 1999), *ptc*, for example, has been proposed to respond only to the maximal levels of Ci-A found in cells nearest the AP boundary, while *dpp* responds to lower levels of Ci-A and also to Ci-R. The broad AP boundary stripe of HHRE-directed expression suggests that the HHRE is highly responsive to Ci-A. Differential response to Ci-R and Ci-A is thought to be controlled by cis-regulatory elements outside the local context (within 100 bp) of Ci binding sites in Hh responsive enhancers (Muller and Basler, 2000). Consistent with this hypothesis, we have identified an element, the REPE, which appears to modulate the response of the HHRE to Ci-A.

Although Ci-A is an essential and important activator, which acts directly through the HHRE, it is unlikely that Ci-A function is sufficient for HHRE activity. Several studies have suggested that signal response elements in enhancers are generally not sufficient to activate gene expression (Barolo and Posakony, 2002). Rather, the transcriptional effectors of signals must act cooperatively with other activators to direct robust expression of target genes. This phenomenon, which has been
termed ‘activator insufficiency’ (Barolo and Posakony, 2002), presumably prevents promiscuous activation of potential target genes. It is likely then, that other sites in the HHRE are required in addition to the Ci sites for expression directed by this element. For example, as the HHRE drives reporter gene expression in the wing and antennal discs (not shown) as well as the leg, we might expect a common factor expressed in all three discs to act through the HHRE in combination with Ci. Alternatively, the enhancer might harbor sites that respond to factors specific to each disc type.

**Dpp signals through the D-h repression element to block Brk-mediated repression**

We have identified a short sequence in the REPE, the CMB, which functions to restrict HHRE expression to a narrow dorsal domain. In this study, we provide strong evidence for the hypothesis that the transcriptional repressor Brk acts through the CMB to repress D-h expression. Although previous studies have shown that brk expression is very low or undetectable in cells near the Dpp source, we observe a genetic requirement for brk in repression of D-h in this region. In addition, overexpression of brk results in a dramatic reduction of D-h-GFP expression, but only mildly affects expression from a D-h-GFP transgene with a compromised Brk binding site.

We also found that Dpp acts through the REPE to block Brk-mediated repression. We propose that high-level Dpp signaling defines the domain of D-h expression within the HHRE-response zone. This idea is supported by the observations that D-h-GFP but not HHRE-GFP expression is dependent on Dpp, indicating that Dpp signals through the REPE, and that elevation of Dpp signaling results in expansion of D-h expression along the AP and DV axes, within the domain of HHRE activity. Our current studies suggest that the function of Dpp in regulation of D-h expression may be limited to repression of brk. Yet, the presence of Mad-binding sites in the CMB suggests a potentially more direct role for activated Mad (act-Mad), the transcriptional mediator of Dpp signaling. Brk has been shown to be a potent competitor of Mad in vitro for binding to overlapping binding sites in Dpp target enhancers (Saller and Bienz, 2001). Hence, a potential role for Mad would be to prevent Brk from binding the CMB, thereby blocking Brk repression in cells receiving high-level Dpp signaling. If this model is correct, we might have expected the MM mutation to compromise D-h expression, which was not the case. However, the destabilization of Brk binding to the MM mutant might have masked a requirement for the Mad sites in blocking Brk repression.

It has recently been shown that an act-Mad/Shn complex represses brk expression by binding a silencer element (Muller et al., 2003). Therefore, as mutation of the Mad sites expands D-h expression, it is possible that Mad acts in concert with Brk through the CMB to repress D-h expression. This notion is not inconsistent with genetic evidence, indicating a requirement for Mad in D-h expression, as loss of Mad function elevates Brk levels, which (as discussed below) can overcome the requirement for CMB-sequences other than the Brk site. However, if this were the case, we might have expected a more severe expansion phenotype with the MM mutant, in which both Brk and Mad binding are compromised. Further analysis is required to determine the role, if any, of the CMB-Mad-binding sites in D-h expression.

**A CRE in the D-h repression element is required for repression**

Given the genetic evidence that Brk represses D-h expression and that Brk binds the CMB element in vitro, the most straightforward hypothesis is that Brk acts directly through the CMB in vivo to repress D-h expression. However, as mutation of the CRE also causes loss of repression, it is formally possible that the CRE rather than the Brk site is important for repression. A potential explanation for this observation is that mutation of the CRE lowers the affinity of this element for binding to Brk, even though the Brk binding site is intact in the CMB-C mutant. Because the levels of Brk in the dorsal leg are limiting, altered affinity could have a significant effect on the level of Brk occupancy of the CMB. However, we observe through EMSA analysis that the CRE mutant CMB binds Brk with an affinity greater than that of the wild-type element (Fig. 3, lanes 23, 24).

As it was not possible to mutate the Brk site without affecting the CRE, the CRE was altered in the BM2 and MBM mutants such that it more closely resembles a canonical CRE. Nevertheless, this change in the CRE may have affected its function. If so, this would be consistent with a model in which the CRE mediates repression of D-h expression, and Brk acts indirectly through the CRE rather than the Brk site. However, the finding that Brk overexpression drastically reduces D-h-C-GFP but not D-h-CB-GFP expression suggests that Brk can act directly through the Brk site, independent of the CRE.

The requirement for CMB-sequences outside the Brk binding site suggests that the context of the Brk site within the CMB is important for repression. A plausible explanation for the requirement of the CRE is that it is bound by a factor, X, which functions to facilitate recruitment of Brk under conditions where Brk levels are limiting, such as in the dorsal leg. Consistent with this hypothesis is the observation that overexpression of Brk greatly reduces D-h-C-GFP expression, suggesting that the requirement for the CRE can be bypassed if the levels of Brk are high enough. However, when Brk levels are limiting, the CRE might contribute more to D-h repression than the Brk site. For example, in the dorsal leg, Factor X might bind the CMB and then form a complex with Brk, relieving the necessity for Brk to bind the CRE directly. This model could explain why D-h expression appears to be significantly more sensitive to Brk-mediated repression than other Brk targets in imaginal discs, such as vestigial (vg) and opomotor-blind (omb). vg and omb are each expressed in broad domains across the center of the wing disc and are repressed by higher levels of Brk than is D-h (Campbell and Tomlinson, 1999; Jazwinska et al., 1999; Minami et al., 1999). Perhaps, the CRE and/or other sequences in the REPE mediate heightened response to Brk. It will be of interest to determine whether other Brk-target genes, such as spalt, which are also repressed by very low levels of Brk, are similarly regulated.

A second potential function for a CRE-binding factor X is to act in concert with Brk to mediate D-h repression. Several lines of evidence suggest that Brk is a versatile repressor, which can inhibit transcription by competing with activators for binding to a common site or by active repression. Active repressors can act either at short range, by inhibiting activity of activators bound to nearby elements (150 bp away or less), or at long range by interfering with activators bound at a greater distances (Cai et al., 1996). Brk can mediate active repression...
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subset of its target genes, whereas neither is required for repression, respectively (Saller et al., 2002; Zhang et al., 2001). Brk requires Gro and/or dCtBP function for repression of a subset of its target genes, whereas neither is required for repression of others (Hasson et al., 2001). In the D-h enhancer, the CMB is positioned about 1 kb from the HHRE, suggesting that CMB-binding repressor(s) act at long range to repress HHRE-directed expression. Although Brk directly binds Gro, factor X could facilitate recruitment of Gro or other co-factors required for long-range repression.

Integration of Dpp and other signals

In this study, we identify a novel function for Brk as repressor of Hh-target gene expression. Brk was originally identified as a repressor of Dpp-target genes (Campbell and Tomlinson, 1999; Jazwinska et al., 1999; Minami et al., 1999) and a recent study indicates that Brk can block Wg-mediated transcription as well (Saller et al., 2002). Brk was shown to antagonize function of a Wg-responsive element in the midgut enhancer of the Ultrabithorax (Ubx). The Ubx midgut enhancer drives Ubx expression in parasegment (ps) 7 of the embryonic midgut (Thuringer et al., 1993). Two elements, one which is Wg responsive (the WRS) and another Dpp responsive (the DRS) function synergistically to activate Ubx expression in ps 7 expression (Yu et al., 1998). In the adjacent ps8, however, Brk binds to the DRS and blocks the activity of the WRS (Saller et al., 2002). Curiously, the D-h-CMB and the Ubx-DRS are similarly organized in that each consists of overlapping CRE/Mad and Brk sites. The Ubx-DRS appears to mediate two modes of signal integration which involve: (1) synergistic activation, in which Mad/Med and dTCF act together to activate expression; and (2) activation and refinement, in which there is Wg mediated activation combined with Brk repression, which is blocked by Dpp. In the D-h enhancer, however, the CMB appears to be a component of a dedicated repression element, which appears to mediate only the second mode of signal integration: activation and refinement. The similar organization of the CMB and DRS suggests that it may be possible to predict the structure of enhancers known to be Brk responsive and which integrate Dpp and a second signal.

Despite the similarities, there are important distinctions between the D-h and Ubx-midgut enhancers, suggesting that the mechanisms of Brk-mediated repression might differ in each case. In the Ubx-midgut enhancer, the DRS and WRS are separated by 10 bp, suggesting that Brk acts at short range to inhibit WRS activity. In the D-h enhancer, however, the CMB is positioned at least 1 kb from the HHRE, implying a long-range effect for this element. Furthermore, Brk repression of the WRS depends on Teashirt (Tsh), which binds Brk and acts as a co-repressor (Saller et al., 2002). Tsh is unlikely to be required for D-h repression because it is only expressed in proximal leg segments (Erkner et al., 1999). Our studies suggest the requirement for a second DNA-bound factor, which binds the CRE, in addition to Brk for repression. The DRS-CRE, however, is required in addition to the Mad-binding sites for activation of Ubx in ps 7 (Eresh et al., 1997; Szuts et al., 1998).

Competing inputs by Ci and Brk define a stripe of hairy expression

Together, our observations are consistent with a model (Fig. 9) in which Ci, acting through the HHRE, activates D-h expression. The domain of HHRE activity can be divided into two zones, 1 and 2 (Fig. 9). The HHRE has the potential to direct expression in both zones 1 and 2, but its activity is restricted to zone 1 by Brk and perhaps a second factor, X, which binds the CRE. In zone 2 cells, Brk would bind to the CMB and repress HHRE-directed expression. We propose that zone 1 is defined by the overlap of Hh and high-level Dpp signaling. Dpp promotes D-h expression by repressing brk expression in zone 1. However, the presence of Mad-binding sites in the CMB suggests the potential for a more direct role for Mad in D-h regulation, perhaps in competing with Brk for binding to the CMB, as shown, or in directly mediating repression. Confirmation of a role for the Mad sites awaits further analysis of the D-h enhancer.

Connecting enhancer function to morphology

We show here that establishment of D-h expression in a defined domain is a complex process. This may be explained in part by the observation that morphological elements such as leg bristle rows are remarkably invariant in position from one individual to the next in Drosophila melanogaster. Hence, precise expression of genes such as h, the function of which is so crucial for positioning of elements such as the leg sensory bristles,
is essential. The organization of the D-h enhancer is reminiscent of that observed in another recently described enhancer, which is necessary for the development of a specific morphological element of an adult appendage. The knirps (kn) second longitudinal wing vein (L2) enhancer drives expression of kni, which is required to initiate L2 development, in a narrow stripe within the L2 primordia (Lunde et al., 2003). As observed with D-h, localized expression of kni in the L2 primordia is established by an enhancer consisting of discrete activation and repression elements. The activation element directs broad expression, which is refined by the repression element. The structure of the kni repression element appears complex in that it is thought to bind a number of repressors, including perhaps Brk. Although, we show here that the CMB element is important and essential for D-h repression, it is likely that there is a greater degree of complexity in the D-h-REPE, as well. Further analysis of the HHRE and REPE should provide mechanistic insight into how activation and repression elements function coordinately to establish precisely defined gene expression.

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