Pattern-specific expression of the Drosophila decapentaplegic gene in imaginal disks is regulated by 3’ cis-regulatory elements

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The pattern of structures on most of the adult cuticle of Drosophila is determined in the larval imaginal disks. The Drosophila growth factor homolog decapentaplegic (dpp) is believed to participate in pattern formation in imaginal disks, primarily along what will become the proximal-to-distal axis of adult appendages. We report that dpp expression in wing, leg, and eye-antennal imaginal disks is localized to a band of cells along the presumptive proximal-to-distal axis. The pattern and level of dpp expression in imaginal disks is affected by mutant lesions that remove 3’ cis-regulatory sequences. We demonstrate that one portion of the 3’ cis-regulatory region contains regulatory elements sufficient to activate gene expression in a subset of the cells that normally express dpp in the imaginal disks, allowing rescue of dpp mutant phenotypes. We propose that the complete dpp expression pattern is generated by an array of 3’ regulatory elements that differ in their potency in specific disks and in certain positions within a disk. The identification of the factors that activate these elements should provide clues as to how positional information is encoded in imaginal disks.

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With the exception of the abdominal cuticle, the entire Drosophila adult cuticle is derived from the 19 imaginal disks. The imaginal disks are folded sheets of epithelial cells that each arise from a cluster of 2–20 cells allocated in the embryo at blastoderm formation (for review, see Postlethwait 1978). These cells undergo at least one mitotic division between 3 and 10 hr after oviposition (Wieschaus and Gehring 1976) and then remain quiescent until late in the first-larval instar or early in the second-larval instar. At this time, rapid cell divisions ensue until the mature disks contain 4,000–50,000 cells late in the third-larval instar. At the onset of metamorphosis, the disks respond to increasing titers of 20-hydroxyecdysone, resulting in a series of cell rearrangements and shape changes that enable the epithelial sheet to unfold, secrete cuticle, and form the adult appendages (Fristrom 1976).

The pattern of structures on the Drosophila adult cuticle is established in the larval imaginal disks by a molecularly undefined system of positional information. The generation of imaginal disk fate maps indicates that at the third-larval instar imaginal disk cells are determined to become specific structures on the adult cuticle (for review, see Bryant 1978). Analysis of marked cell clones indicates that cell lineage is not the determining factor in imaginal disk pattern formation. Experiments on the regenerative properties of imaginal disk fragments led to proposals that systems of positional information or positional coordinates within imaginal disks specify the fate of cells (for review, see Meinhardt 1982).

Genes that participate in specifying cell fates in imaginal disks may be expected to exhibit spatially restricted patterns of expression in imaginal disks and, when mutated, to cause a loss or duplication of imaginal disk-derived structures. Several genes are known to have spatially restricted patterns in the imaginal disks including the homeotic genes Ultrabithorax, Antennapedia, engrailed (en), the integrin homolog PS2, and the secreted proto-oncogene homolog wingless (Brower et al. 1984; Kornberg et al. 1985; White and Wilcox 1985; Wirz et al. 1985; Baker 1988). One class of mutant alleles of the decapentaplegic (dpp) gene causes phenotypic defects in imaginal disk-derived structures (for reviews, see Gelbart 1989; Hoffmann 1990). The product of dpp is homologous in sequence and biochemical properties to the secreted proteins in the transforming growth factor-β (TGF-β) family of growth factors (Padgett et al. 1987; Panganiban et al. 1990). In agreement with the biochemical characterization of dpp, dpp function in the wing imaginal disk is cell nonautonomous, that is, the phenotype of cells mutant for dpp is rescued by dpp-expressing cells elsewhere in the same wing imaginal disk (Spencer 1984; Posakony 1987; Posakony et al. 1990).

Although null mutations at dpp are embryonic lethals
causing defective gastrulation and dorsal–ventral polarity (Irish and Gelbart 1987), the 30 dpp disk alleles result in the absence of adult cuticular structures along the proximal-to-distal axis of adult appendages (Spencer et al. 1982). The dpp disk alleles map from 2 to 26 kb, 3' of the transcribed region of dpp, indicating the presence of cis-regulatory sequences required for imaginal disk expression of dpp (St. Johnston et al. 1990). With the exception of two internal deletions of the dpp disk region, all of the dpp disk alleles are chromosomal rearrangements. The mildest alleles map farthest from the transcribed region of dpp and result in the absence of structures on either the wing blade or the eye. More severe alleles map closer to the transcribed region and affect more structures derived from all of the imaginal disks. The effects of the dpp disk mutations can be seen in the imaginal disks, which are smaller and show signs of defective cell proliferation and extensive cell death (Bryant 1988).

To understand the functions of the 3′ regulatory region in directing dpp expression in imaginal disks, we have determined the patterns of wild-type dpp expression in the disks from third-instar larvae and early pupae, as well as dpp expression in disks homozygous for mild dpp mutations. We demonstrate that dpp is expressed in cells just anterior to the anterior–posterior compartment boundary in the wing and leg imaginal disks, in the morphogenetic furrow and periphery of the eye disk, and in a medial-to-lateral stripe of the antennal disk. Upon evagination of the disks, this expression follows the proximal-to-distal axis of adult appendages. Mutations reduce expression in specific disks and in specific regions of the disks. We provide evidence that the 3′ regulatory sequences induce pattern-specific expression from either a dpp promoter or a heterologous promoter. The expression of dpp from the transposon construct is sufficient to rescue the dpp mutant phenotypes.

Results

Wild-type pattern of imaginal disk dpp expression

Digoxigenin-labeled DNA probes were used to detect dpp mRNA in whole-mount imaginal disks of third-instar larvae. dpp mRNA was detected in specific subsets of imaginal disk cells (Fig. 1). The earliest disks examined were from animals at the early third-instar larval stage (Fig. 1a,d,g). Detectable dpp expression is restricted to the primordia of the ventral aspect of the wing and the medial side of the leg (Fig. 1a,d, arrows). As the disks mature, the number of cells expressing dpp mRNA increases to form a discontinuous stripe across the wing and leg imaginal disks (Fig. 1b,e). Late in the third-larval instar, another stripe of staining is detected along the posterior edge of the wing disk (Fig. 1c, arrowhead). In contrast to the band of expression across the wing disk, dpp expression in the leg disk is more intense in the region that becomes the lateral side of the leg (Fig. 1f, ls).

In the early third-instar eye imaginal disk, the stripe of dpp-expressing cells extends around the periphery of the eye disk primordia (Fig. 1g). As the disk matures, the stripe of dpp expression along the posterior portion of the disk migrates with the morphogenetic furrow while the lateral and medial stripes of expression remain stationary (Fig. 1h). As in the leg imaginal disk, the stripe of cells expressing dpp on the lateral edge of the eye disk is more intense than that on the medial edge (Fig. 1i). The cells expressing dpp in the early third-instar antennal disk are situated in the centrolateral portion of the disk (Fig. 1g, arrow). It becomes apparent in older disks that this expression is on the lateral side of the presumptive antennal segments. Also at this stage, faint expression is detected across the medial edge of each antennal segment. In late third-instar disks, two additional stripes are detected in the antennal disk (Fig. 1i, arrowheads).

dpp expression is adjacent to the compartment boundary

Expression of the segment polarity gene en is localized to the posterior compartments of the leg and wing imaginal disks (Kornberg et al. 1985). Digoxigenin-labeled probes for dpp (Fig. 2c,f) and en (Fig. 2a,d) were used separately and in the same disks (Fig. 2b,e) to compare the location of dpp-expressing cells with respect to the anterior–posterior compartment boundary defined by en expression. The results indicate that dpp is expressed in cells just anterior to the cells expressing en. This experiment does not rule out that some cells at the boundary coinpress dpp and en. Consistent with these localization results, analysis of dpp mutant clones in wing disks has led to the conclusion that expression of dpp anterior to the compartment boundary is critical for dpp function in the disk (Posakony 1987, Posakony et al. 1990).

dpp expression persists during the initial stages of disk eversion

At the onset of metamorphosis, the wing and leg imaginal disks evaginate and take on the shape of the adult wing and leg appendages while the two sets of eye–antennal disks fuse to form the head capsule. The localization of dpp mRNA was determined in imaginal disks from white prepupae (Fig. 3a,d,g), and prepupae at 3.5 hr (Fig. 3b,e,h) and 5.5 hr (Fig. 3c,f,i) postpuparium formation. Staining seen in the mature third-instar disks persists through the initial stages of eversion. Additional expression of dpp is detected in the anterior region of the wing pouch of white prepupae (Fig. 3a, as). In the evverting wing and leg imaginal disks (Fig. 3c,f), the previously discontinuous spots of expression detected in the third-instar imaginal disks (Fig. 1c,f) are revealed as continuous stripes along the proximal–distal axis. The expressing cells are present on both the dorsal and ventral surfaces of the wing blade; however, in evverting leg disks, the lateral stripe of expression (Fig. 3f, ls) is more intense than the medial stripe (Fig. 3f, ms).

All expression detected in the eye–antennal disks prior to puparium formation (Fig. 3g) persists as the two sets of disks fuse (Fig. 3h,i). The anterior-most cells of the eye, which are the last cells to enter the morphoge-
Figure 1. Wild-type dpp expression in imaginal disks at three stages of the developing third-larval instar. Imaginal disks from early (left), intermediate (center), and late (right) third-instar larvae were analyzed for expression of dpp RNA. (a–c) Developing wing disks are oriented anterior to the left and ventral to the top. dpp expression at early third instar is restricted to the ventral-most region of the wing disk (a, arrow). Later, dpp expression extends discontinuously across the center of the entire wing disk and appears at the posterior edge (c, arrowhead). Regions of the disk are indicated as follows: (ap) anterior border of wing pouch; (pp) posterior border of wing pouch; (dwb) distal tip of wing blade; (vh) ventral hinge structures; (dh) dorsal hinge structures; (scu) scutellum. (d–f) Developing leg disks are oriented anterior to the left and lateral side to the top. dpp expression at early third instar is restricted to the medial side of the leg disk (d, arrow). The expression evolves into a discontinuous stripe across the entire disk (e, f). The lateral stripe of dpp expression (ls in f) is more prominent than the medial stripe (ms in f). Fate map position of tarsal claws (tc) is indicated. (g–i) Developing eye–antennal disks are oriented lateral side to the left and posterior to the top. Eye primordia is on top; antennal primordia is at bottom. dpp expression in the early third-instar eye disk surrounds the periphery (g) and, in the antennal portion of the disk, is localized to the lateral side of the disk (g, arrow). The posterior limit of eye disk expression travels with the morphogenetic furrow (mf in i). Lateral and medial stripes of eye disk expression (ls and ms in i) extend anteriorly into the antennal disk. dpp expression on the lateral side of the antennal disk is maintained in later disks (h, i). Additional dpp expression is detected in late third-instar antennal disks in two stripes (i, arrowheads) and across the medial side of the antennal segments. Regions of the disk are indicated as follows: (ar) aristae; (an3) third antennal segment; (an2) second antennal segment; (an1) first antennal segment.
netic furrow, continue expressing dpp after puparium formation. The result is a ring of expression around the border of the developing eye. Furthermore, cells that express dpp in the lateral (Fig. 3g, ls) and medial stripes (Fig. 3g, ms) of the eye in late third-instar disks continue to express dpp, although the medial stripe broadens by 3.5 hr postpuparium formation (Fig. 3h). The cells expressing dpp in the everting antennae form a continuous stripe along the lateral side of the antennae (Fig. 3i, arrowhead). The faint medial stripe is not detected in the everting antennal disk.

dpp mutations decrease dpp mRNA expression

Mutations that specifically affect dpp function in the imaginal disks map in a 3'-nontranscribed region proposed to contain cis-regulatory sequences (St. Johnston et al. 1990). Most of these mutations are chromosomal rearrangements that break the DNA in the 3’ regulatory region and separate the putative regulatory sequences on the proximal side of the break from the dpp transcriptional unit on the distal side of the break. Because the imaginal disks from larvae mutant for strong dpp^{disk} alleles are too small to be readily dissected, imaginal disks from larvae homozygous for three of the mildest dpp^{disk} mutations, dpp^{lp}, dpp^{ts}, or dpp^{ls}, were probed with digoxigenin-labeled DNA probes. The mutation dpp^{lp} is a 3-kb deletion of sequences 23 kb 3’ of the dpp transcriptional unit (Blackman et al. 1987). dpp^{ts} causes a held-out wing and defects in a sensory structure on the wing blade, the sensilla campaniformia 25 (sc25; Spencer et al. 1982). The dpp^{ts} mutation is a DNA rearrangement with one breakpoint 21 kb 3’ of the dpp transcriptional unit (St. Johnston et al. 1990), which reduces the size of the wing blade and slightly reduces the size of the eye. Both mutations reduce the level of dpp expression in the mutant wing disks (Fig. 4b,c), as compared to a wild-type disk (Fig. 4a). The area of reduced expression seen in the wing disk of dpp^{lp} mutants corresponds to the fate map position of the sc25. dpp expression in the dpp^{ts} mutant wing disk is reduced along the entire anterior-posterior compartment boundary, resulting in only five localized spots of expression rather than the discontinuous stripe of dpp expression observed in wild-type disks. In dpp^{ts} eye disks, which form a slightly reduced eye, the level of dpp expression along the morphogenetic
Figure 3. Wild-type dpp expression during imaginal disk eversion. Orientations and abbreviations are as in Fig. 1. Everting wing (a–c), leg (d–f), and eye–antennal (g–i) disks from white prepupae (left), animals 3.5 hr postpuparium formation (center), and animals 5.5 hr postpuparium formation (right) were analyzed for expression of dpp RNA. Comparison of the patterns of expression in disks beginning to evert (a,d,g) with mature third-instar disks (shown in Fig. 1c,f,i) indicates that most dpp expression persists unchanged during the initial stages of eversion. During eversion, a novel stripe of expression is detected in the anterior portion of the wing disk (arrow at as). In the everting wing and leg disks (b,c and e,f) the stripe of dpp expression in the disk is resolved into a band of expression along the proximal-to-distal axis of the appendages. The distal end of the wing blade is up in b and c, and the distal end of the leg is down in e and f. Two sets of eye–antennal disks fuse along their medial sides to form the head capsule (h,i). Antennae are everting into the plane of the page (for further details, see text).
Figure 4. dpp expression in mild dpp disk mutants that specifically affects wing development. Orientations are as in Fig. 1. Imaginal disks from late third-instar larvae, either wild type [left] or homozygous for the mutations dpp^bho (center) or dpp^bs (right), were analyzed for dpp RNA expression. Disks were processed in parallel. When the wild-type pattern of dpp expression in the wing disk [a] is compared to the dpp expression in a dpp^bho mutant wing disk [b], a reduction in expression is apparent in the dorsal hinge area of the mutant disk. This is the same region of the wing disk that gives rise to the sensilla campaniforme 25 (sc25 in a,b), the structure that is missing in dpp^bho mutant wings. [c] dpp^bs substantially reduces the intensity of dpp expression across the mutant wing disk such that only a subset of the wild-type pattern is detected. [d-f] Neither mutation affects dpp expression in the leg disks.

The mutation dpp^bho is a 5-kb deletion of sequences 17 kb 3' of the dpp transcribed region [Blackman et al. 1987]. dpp^bho causes a reduction in the number of eye facets from the wild-type complement of 700 [Fig. 5c] to ~100–200 [Fig. 5a] but does not affect any other imaginal disk-derived structures. Each ommatidium in the mutant eye exhibits a full complement of photoreceptor cells that are patterned normally. The axis of dorsal–ventral symmetry that normally bisects the ommatidial clusters [Fig. 5e, arrow] is asymmetrically positioned in the dpp^bho eye [Fig. 5d], where all but a few ventral ommatidia are missing. Examination of cell death in the dpp^bho mutant eye disks by accumulation of acridine orange indicates that cell death is more prevalent on the ventral side of the mutant eye disk [Fig. 6a, arrow] when compared to a wild-type disk [Fig. 6b]. Also apparent in Figure 6 is the reduced size of the eye disk in dpp^bho mutant animals, indicating that decreased cell proliferation and/or increased cell death probably occur throughout the development of the eye disk. dpp expression in the dpp^bho mutant eye disks [Fig. 7a] is reduced below the level detectable with a digoxigenin-labeled DNA probe that readily detects dpp RNA in wild-type eye disks [Fig. 1] and in leg disks [Fig. 7d] derived from dpp^bik mutant larvae.

dpp disk region sequences impart imaginal disk expression to the dpp and alcohol dehydrogenase promoters

A 4-kb BamHI fragment spanning 106–110 of the dpp disk region was inserted into a P-element transposon that contained a dpp promoter and protein-coding exons; the alcohol dehydrogenase (adh) gene was present on the transposon as a selectable marker [Fig. 8a]. The 106–110 BamHI fragment in Tn|MB7 is located just 5' to the adh
promoter. A transgenic line was established with transposon TnJMB7. Digoxigenin-labeled DNA probes for adh were used to determine whether the 4-kb dpp fragment affected the expression of the heterologous adh promoter. adh mRNA was detectable in cells in the eye disk (Fig. 7c) and also in the wing [not shown] and leg imaginal disks [Fig. 7f] of transgenic animals in patterns similar to parts of the wild-type pattern of dpp-expressing cells [Fig. 1f,i]. adh RNA expressed from an independently derived transposon line of a similar construct [Fig. 8b] was detected in a pattern indistinguishable from that seen with TnJMB7 [data not shown]. The identical patterns of adh expression in the disks provided by both transposons argue against the existence of artifactual expression due to site of insertion. No adh mRNA was detected in imaginal disks from control flies that were transgenic for the dpp P element lacking the disk sequences [data not shown].

A P-element transposon lacking the 106-110 fragment but containing the dpp promoter and protein-coding exons [Hin region] has no effect on dpp disk mutations [Hoffmann and Goodman 1987]. Similarly, a transposon containing only 106-110 has no effect on dpp disk mutant phenotypes, indicating a cis-acting mechanism [data not shown]. The TnJMB7 transposon was tested for its ability to rescue the dpp disk mutation. One copy of the transposon was sufficient to rescue the phenotype of dpp disk [Fig. 5b] to wild type. Eye disks from dpp disk larva carrying two copies of TnJMB7 in the genome were examined for dpp expression and found to have detectable dpp expression in a pattern indistinguishable from wild type [Fig. 7b]. The eye-specific defects caused by the deletion of the 106-110 region in dpp disk and the rescue of the eye phenotype by TnJMB7 are consistent

Figure 5. Adult eye phenotypes of dpp disk mutants and transposon-rescued dpp disk mutants. Anterior is to the left and dorsal is to the top. (a) Approximately 100–200 ommatidia develop in the dorsal region of the dpp disk mutant eye. (b) One copy of TnJMB7 rescues the dpp disk mutant eye phenotype to near wild-type number of ommatidia and fully restores a normal, highly ordered array to the ommatidia. (c) A wild-type eye is made up of ~700 ommatidia. (d,e) Equatorial cross sections of adult eyes reveal the predominant dorsal orientation of ommatidia in dpp disk mutants (d) compared to wild type (e). Each ommatidium contains a full complement of photoreceptor cells, which are organized in a trapezoidal array. The trapezoids point in opposite directions on either side of the equator (arrows), which divides the wild-type eye into equal dorsal and ventral halves. In the dpp disk mutant eye, only a few ventrally oriented ommatidia exist.

Figure 6. Acridine orange staining of dpp disk mutant and wild-type eye–antennal disks. Disks are oriented anterior left and dorsal to the top. Unfixed, late third-instar eye–antennal disks from dpp disk mutant (a) and wild type (b) were stained with the fluorescent dye acridine orange to mark regions of cell death. dpp disk mutant eye disks are aberrantly shaped and smaller than wild type. A stripe of fluorescence just anterior to the morphogenetic furrow is common to both mutant and wild-type eye disks. In the dpp disk mutant eye disk, a novel patch of intense fluorescence is detected on the ventral side [arrow].
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Figure 7. RNA expression in imaginal disks of dpp^d-b^k mutants and dpp^d-b^k mutants rescued by TnJMBZ. Orientations are as in Fig. 1. Imaginal disks from dpp^d-b^k mutants (left) and dpp^d-b^k mutants transgenic for two copies of TnJMBZ (center and right) were analyzed for expression of dpp RNA (left and center) or adh RNA (right). [For wild-type expression of dpp, refer to Fig. 1.] No adh expression is detected in imaginal disks derived from larvae lacking the transposon [data not shown]. (a,d) No dpp expression is detectable in the morphogenic furrow (mf) of dpp^d-b^k mutant eye disks, but antennal and leg disk expression is wild type. (b,e) Two copies of TnJMBZ restore dpp expression in the morphogenic furrow of dpp^d-b^k mutant eye disks; the dpp expression in the leg and antennal disks is the sum of the expression from the endogenous gene and the transposon. (c,f) TnJMBZ drives adh expression in a subset of the positions normally expressing dpp.

with the presence of regulatory elements in the 106–110 fragment that are necessary for dpp expression in the eye imaginal disk. No ectopic expression from TnJMBZ was detected [Fig. 7e].

Rescue of dpp mutant phenotypes in other disks by TnJMBZ

TnJMBZ was crossed into dpp mutant backgrounds exhibiting defects in almost all imaginal disk-derived structures. Chromosomal rearrangement breakpoints of the class III mutations lie 11–20 kb 3' of the transcribed region [100–109 on the dpp genomic map; Fig. 8] and separate all of the regulatory sequences beyond the breakpoint from the dpp transcribed region. These alleles cause defects in all imaginal disk-derived portions of the adult cuticle; for example, the wing blade is reduced to a stump [Fig. 9a]. One copy of TnJMBZ provided sufficient dpp expression to completely rescue the eye defects of the class III allele dpp^d6 (not shown) and partially rescue leg, antennae, haltere, and wing [Fig. 9b] defects. Two copies of TnJMBZ allowed full phenotypic rescue of class III (dpp^d6) eye, leg, antennae, and haltere defects and substantial rescue of the wing defects as well [Fig. 9c]. These animals are fertile and can be maintained as a stock. Class V mutations are chromosomal rearrangements that remove all of the dpp^disk regulatory elements >2 kb 3' of the dpp-transcribed region. The mutant animals die as early pupae due to the absence of adult cuticle derived from the imaginal disks. With two copies of TnJMBZ, 35–40% of the mutant animals (dpp^dfe/dpp^dfe) form a full adult cuticle, but most of these fail to eclose. Those that do eclose from the pupal case [4–8% of the mutant progeny class] have normal
eyes, some complete legs and some legs missing tarsal segments, and near full-sized, uninflated wings (not shown).

**Discussion**

Specific positions in imaginal disks correspond to specific positions and structures on the adult cuticle. The mechanisms by which a field of positional information is established and maintained in each imaginal disk are unknown. We have described the highly localized patterns of dpp expression in the imaginal disks and established that the 3'-nontranscribed region of dpp is necessary for proper expression in specific cells within imaginal disks. Our results indicate that dpp expression is associated with the hypothetical proximal-to-distal axis of imaginal disk positional information. The mutant phenotypes of the dpp<sup>disk</sup> alleles also support a role for dpp along the presumptive proximal-to-distal axis; however, it remains to be determined whether the role of dpp is in establishing, maintaining, or transmitting imaginal disk positional information.

We have shown that dpp mutations, which disrupt imaginal disk development, reduce the level or affect the pattern of dpp expression in the imaginal disks. In the earliest disks examined from early third-larval instar, dpp expression is already localized to specific regions of the disks; we suspect that dpp may be localized even earlier and required throughout disk development. The pattern of dpp expression in the leg and wing disks evolves as the disk cells proliferate during the third-larval instar and is maintained through the initial stages of disk eversion. The small size and altered morphology of imaginal disks caused by severe (class V) dpp<sup>+++</sup> mutations indicate that dpp is required throughout disk development for cell proliferation and/or cell viability.

The earliest known event in establishing domains within imaginal disks is the formation of the anterior–posterior compartment boundary in the disks derived from the metameric regions of the embryo (for review, see Brower 1985). These cell lineage boundaries in the disks may correspond to the parasegment boundaries established at blastoderm when the disk primordia are set aside. Localized expression of dpp transcripts is detected in head and thoracic segments of the embryo at positions corresponding to the imaginal disk primordia; this expression responds to segment polarity pattern formation genes that might be involved in localizing dpp adjacent to the anterior–posterior compartment boundary (P.D. Jackson and F.M. Hoffmann, in prep.). Localization of dpp expression relative to en reported here, and analysis of clones of dpp mutant cells [Posakony et al. 1990]
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Figure 9. Progressive rescue of dpp<sup>dis</sup> mutant wing phenotype with increasing copies of TnJMB7. Adult dorsal wing surfaces are shown at the same magnification. (a) dpp<sup>dis</sup> class III mutants develop wing stumps that lack venation. (b) One copy of TnJMB7 increases the size of dpp<sup>dis</sup> mutant wings. (c) Two copies of TnJMB7 rescue the mutant wing to 80% wild-type size. Defects along the third longitudinal wing vein are observed in all genetic backgrounds homozygous for the transposon.

both support the conclusion that a band of cells immediately anterior to the compartment boundary express dpp. We believe that the compartment boundary may be used to orient dpp expression along the presumptive proximal-to-distal axis of the wing and leg disks. The eye-antennal disk is derived from a nonmetameric portion of the embryo and does not exhibit the anterior-posterior cell lineage boundary until the third-larval instar (Morata and Lawrence 1978). Therefore, early expression in the eye-antennal disk of dpp may be regulated by other positional cues.

Some portions of the dpp<sup>disk</sup> regulatory region are particularly potent in specific imaginal disks. This is illustrated by the eye disk-specific defects of dpp<sup>d-bik</sup>, the wing disk-specific defects of dpp<sup>d-be</sup>, and the wing and eye disk-specific defects of dpp<sup>ds</sup>. The dpp<sup>d-bik</sup> mutation specifically reduces the level of dpp expression in the eye disk to below our limits of detection; either there must be some residual dpp expression or dpp expression prior to third instar in the dpp<sup>d-bik</sup> eye disk because the dpp<sup>d-bik</sup> mutant phenotype in the eye is not as severe as that caused by class III and class V dpp mutations. The dpp<sup>d-bik</sup> mutation does not cause any detectable change in the intensity of staining in disks other than the eye disk. Similarly, the dpp<sup>ds</sup> mutation reduces the level of dpp expression across the wing disk and somewhat in the eye disk but has no detectable effect on the level of expression in the leg disk, at least as deduced by the relative intensity of staining in RNA in situ hybridizations.

Although the regulatory elements affected by dpp<sup>d-bik</sup> are extremely potent in the eye and the regulatory elements removed by dpp<sup>ds</sup> are most critical in the wing disk, these two regions of dpp regulatory elements are not disk-specific. Mutations like dpp<sup>ds</sup> (for map positions of alleles, see Fig. 8), which remove the dpp regulatory elements in 106–110 and the regulatory elements beyond position 110, cause severe defects in all imaginal disk-derived structures. In contrast, mutations like dpp<sup>ds</sup>, which remove only the regulatory elements beyond position 110 and leave the 106–110 region contiguous with the rest of the dpp, cause only moderate wing defects and mild eye defects. The presence of the regulatory elements in the 106–110 region must provide dpp expression in all disks to account for the milder phenotype of dpp<sup>d-bik</sup>. This is consistent with our observation that the 106–110 fragment drives adh expression in the leg and wing disks, as well as in the eye disk. Deletion of the elements on the 106–110 fragment by dpp<sup>d-bik</sup>, however, does not affect the development of disks other than the eye disk. This indicates that the regulatory elements beyond position 110, removed by both dpp<sup>ds</sup> and dpp<sup>d-bik</sup>, but not by dpp<sup>d-bik</sup> must also function in all disks. In support of this hypothesis, it has been reported that different disks use the same system of positional information (Bryant et al. 1978). Therefore, neither region is disk-specific, but each is likely to be position-specific. It is important to note, however, that specific regulatory regions, e.g., the 106–110 region, can be more or less critical to the development of specific disks. This is illustrated by the ability of one copy of TnJMB7, containing only the regulatory sequences in the 106–110 interval, to fully rescue the eye phenotype not only of dpp<sup>d-bik</sup>, but also of dpp<sup>ds</sup>. We conclude that the 106–110 regulatory region may not be eye disk-specific, but it is extremely potent in the eye disk.

Individual dpp<sup>disk</sup> region regulatory elements may respond to specific positions within a disk. Analysis of the dpp<sup>d-bik</sup> phenotype indicates that deletion of part of the dpp regulatory region specifically debilitating development in the ventral half of the eye disk, leading to ventrally localized cell death and the absence of ventrally oriented retinal cell clusters. Presumably, other dpp reg-
ulotary elements not affected by dppΔ^disk are active in the dorsal half of the eye disk. Similarly, the regulatory elements removed by the dppΔ^{180} mutation lead to a reduction in dpp expression only near the center of the wing disk and the absence of an adult cuticular structure derived from the center of the disk. In the leg and antennal disks, some of the dpp regulatory elements must discriminate between medial and lateral halves of the disks to provide much higher levels of expression in the lateral half.

Multiple regulatory regions in the dpp^disk region are needed to provide the complete pattern of dpp expression in any one disk, because individual regions appear to contribute only specific portions of the complete pattern. This is illustrated by position-specific reductions in expression caused by dpp mutant alleles. Consistent with this, adh expression from TnJMB7 and TnJMB2 is in only a subset of the positions in the leg disk that express dpp. We predict that portions of the regulatory region on either side of the 106–110 fragment are responsible for the expression of dpp in other parts of the disk.

The requirement for two copies of the dpp gene for proper embryonic development indicates that embryonic cells require a certain level of dpp expression (Irish and Gelbart 1987). Our observations indicate that imaginal disk cells are sensitive to the level of dpp expression as well. For example, there is a significant improvement in the dppΔ^disk wing blade (Fig. 9b,c) when the dose of TnJMB7 is increased from one copy to two copies. Two copies of TnJMB7 also improve the phenotype of dppΔ^{12}/dppΔ^{24} [class V] mutant animals significantly more than one copy of TnJMB7. The dppΔ^{12}/dppΔ^{24} mutant animals are not rescued as well as the dppΔ^{26} [class III] mutant animals, because the class V alleles remove even more of the dpp 3′ regulatory region. Perhaps three or four copies of TnJMB7 would provide more complete rescue of the class V phenotype. Apparently, the incomplete set of regulatory sequences on TnJMB7 can be partially compensated for by higher levels of dpp expression in the wing disk achieved by two copies of the transposon. The compensatory effect of higher levels of dpp expression in a subset of the cells that normally express dpp may simply reflect the cell-nonautonomous property of the secreted dpp protein. dpp protein is secreted in S2 cells (Panganiban et al. 1990) and in embryos (G.E.F. Panganiban, R. Reuter, M.P. Scott, and F.M. Hoffman, in prep.), it is likely to be secreted in the imaginal disks as well. It is not known to what extent dpp diffuses in imaginal disks, but the central location of its expression could allow most, if not all, of the cells of the disk to be exposed to the dpp product, perhaps in a graded fashion. To more fully understand the function of dpp in the disks, it will be important to determine the distribution of dpp protein in the disk. The properties of the protein in culture (Panganiban et al. 1990b) and in the embryo (G.E.F. Panganiban, R. Reuter, M.P. Scott, and F.M. Hoffman, in prep.) lead us to predict that the secreted dpp protein will be found in a fairly limited area due to association with other extracellular proteins.

We propose that specific disks and specific regions within a single imaginal disk may have different requirements for dpp product, which are effectively met by an array of regulatory elements. These elements may differ in how well they function in different disks, either because they respond particularly well to disk-specific trans-acting factors or because they provide dpp expression at a position in a disk where its function is particularly critical for the development of that disk. Further dissection of the dpp^disk region should distinguish between these possibilities. It is not yet clear what the role of dpp is in imaginal disk development and why its expression is so exquisitely localized. It will be important to determine whether localized expression of dpp is essential to its role in disk development. We are particularly interested in the proteins that act on the 3′ cis-regulatory elements to cause expression of dpp in these specific patterns. Identification of these molecules should bring us one step closer to understanding the molecular basis of positional information in the imaginal disks.

Materials and methods

Drosophila cultivating

Drosophila stocks were grown on standard corn meal/sugar/yeast media at 25°C. dppΔ^{180}, dppΔ^{186}, dppΔ^{26}, dppΔ^{24}, and dppΔ^{14} are as described (Spencer et al. 1982, St. Johnston et al. 1990). All other stocks are described in Lindsley and Zimm (1985, 1990).

Digoxigenin labeling of DNA probes for RNA in situ

A modified method of Tautz and Pfeiffer (1989) was used to label the DNA fragments for whole-mount in situ. The 3-kb EcoRI fragment or the 2-kb EcoRI/Apal fragment of the dpp cDNA E55 was used to detect dpp mRNA. These fragments recognize the exons common to each transcript and were used interchangeably. The 4.5-kb EcoRI fragment from the genomic clone pen4.5R (from Dr. Allen Laughon, University of Wisconsin, Madison), which spans the first exon and the first intron, was used to detect the en transcripts. The 2.5-kb Sall fragment of the transposon vector pPA1 (Spradling 1986; gift of Dr. James Posakony, University of California, San Diego), which covers most of the adh gene fragment present in the vector, was used to detect the adh transcripts. Each digest was digested in a volume of 100 μl with HaeII and HaeIII to generate small fragments, phenol/chloroform-extracted, diluted to 2 μl with ddH2O, concentrated on a Centricron 10 column (Amicon) to 50 μl, washed in ddH2O again, and concentrated again on a Centricron 10 column to 50 μl. The DNA concentration was determined by spectrophotometry. Approximately 500 ng of DNA was boiled for 10 min in 10 μl 1 X Vogel buffer [95 mM PIPES (pH 6.5), 5 mM MgCl2, 10 mM β-mercaptoethanol], in the presence of 100 μg pdN6 [Pharmacia], and placed on ice. The tube was spun briefly, and the following additions were made: 1 μl 10 X Vogel buffer, 2 μl 10 X nucleotide mix [1 mM dATP, 1 mM dGTP, 1 mM dCTP, 1 mM dTTP, 0.65 mM dUTP, 0.35 mM digoxigenin dUTP [Boehringer Mannheim Biochemicals] or Genius kit buffer 6 [Boehringer Mannheim Biochemicals], 1 μl Klenow [2U/μl], and 6 μl H2O. After incubation at 16°C overnight, the reaction was boiled for 10 min and chilled on ice. One micro-liter of 10 X Vogel buffer, 1 μl 10 X nucleotide mix, 1 μl Klenow, and 7 μl H2O were added, and the reaction was placed at 37°C for 4 hr. The reaction was stopped by adding 4 μl 0.5 M EDTA and heating to 80°C for 15 min. Fifty micrograms of
RNA in situ on whole-mount imaginal disks

Some modifications were made to a protocol provided by Drs. W. Biggs and L. Zipursky (UCLA). Disks of various stages were dissected into PBS for 15 min or less to remove salivary glands, internal organs, and fat tissue (disks remained attached to mouth hooks and cuticle). Various stages of third-instar disks were dissected en masse and later staged by comparing to photographs in Bodenstein [1950]. Everted disks were staged by picking white prepupa and allowing them to age 3.5 and 5.5 hr at 25°C prior to dissection. The disks were then fixed for 15–20 min in 4% paraformaldehyde in PBS on ice, fixed for 15–20 min in 4% paraformaldehyde, 0.6% Triton in PBS, at room temperature, washed three times for 5 min each in PBT, digested for 3–5 min with 10 µg/ml proteinase K, washed two times for 5 min each in PBT plus 2 mg/ml glycine, washed three times for 5 min each with PBT, fixed 15 min in 4% paraformaldehyde + 0.2% glutaraldehyde in PBS at room temperature, washed five times for 5 min each in PBT, washed 10 min in 50:50 mix of PBT/hybridization buffer (50% denoized formamide, 5 × SSC, 100 µg/ml sonicated, boiled salmon sperm DNA, 200 µg/ml wheat germ tRNA, 0.1% Tween 20) at room temperature, washed for 10 min in hybridization buffer at room temperature, and incubated in hybridization buffer at 48°C for at least 1 hr. Forty nanograms of heat-denatured digoxigenin-labeled probe was added to the prehybridizing disks such that the final volume was ~100 µl. In the dpp/en double-labeling, 10 ng of dpp probe and 40 ng of en probe were used. Hybridizations were allowed to continue for 36 hr at 48°C. Excess probe was washed off by a 20-min wash in hybridization buffer, a 20-min wash in 50:50 PBT/hybridization buffer, and 10–12 hr of washes in PBT with about five changes of buffer. The tubes with the disks in PBT buffer were then placed on ice overnight. The disks were incubated for 1.5 hr at room temperature with 1 ml of a 1:2000 dilution of antidigoxigenin antibody [Genius kit] preabsorbed for 1 hr to crushed, fixed larvae. The disks were then washed four times for 2 hr in PBT and washed for 15 min in staining buffer [100 mM NaCl, 50 mM MgCl₂, 100 mM Tris at pH 9.5, 1 mM Levamisol, 0.1% Tween 20]. The staining reaction was initiated by adding 1 ml staining buffer, 4.5 µl NBT, and 3.5 µl X-phosphate [Genius kit] and incubated for 2–6 hr at room temperature. The reaction was stopped by several washes in PBT, and the disks were dissected and mounted in 80% glycerol.

Acridine orange staining

Some modifications were made to the protocol of Spreij [1971]. Eye-antennal disks were dissected from wandering third-instar larvae in Drosophila Ringer’s solution [128 mM NaCl, 2 mM KCl, 35.5 mM sucrose, 5 mM HEPES, 1.8 mM CaCl₂, 4 mM MgCl₂ at pH 7.1] and stained with 1.6 × 10⁻⁶ M acridine orange [Aldrich] in Ringer’s solution for 5 min. After washing three times briefly in Ringer’s solution, the disks were mounted in Ringer’s solution and viewed immediately by fluorescence microscopy.

Creating transgenic fly lines with JMB2 and JMB7

Transgenic fly lines were created by the method of Spradling [1986]. TnJMB2 and TnJMB7 were made in the P-element vector pPA1, which contains the adh gene as a selectable marker. The defective P-elements Δ2,3 [Robertson et al. 1988] was used to integrate the transposons. TnJMB7 is inserted on the second chromosome, and TnJMB2 is inserted onto TM2.

Transposon rescue of dpp mutant phenotypes

TnJMB7 was recombined onto the following dpp mutant chromosomes: dppΔ1,dppΔ2 adhneo pr cn, dppΔ1 adhneo pr cn, dppΔ2 adhneo pr cn, and dppΔ2 adhneo pr cn. Isogenic recombinant chromosomes were balanced over CyOab and tested for rescue of dpp mutant phenotypes.

Sections of Drosophila eyes

Drosophila eyes were prepared for sectioning as described by Ready et al. [1976]. Sections were cut on a Reichert–Jung ultramicrotome and stained with toluidine blue for light microscopy.

Scanning electron microscopy

Adult Drosophila were dissected mid-thorax, rinsed in PBS, and fixed in 2.5% glutaraldehyde, 2% paraformaldehyde in 0.1% NaPO₄ (pH 7.0) for 30 min to overnight at 4°C. The samples were rinsed three times in 0.1 M NaPO₄ and successively dehydrated in ethanol steps of 35%, 50%, 70%, and 85%, two steps of 95%, and three steps of 100% for 5 min each. Samples were dehydrated further with liquid CO₂ in a Tousimis Sandri-780A critical point drier, mounted onto studs with conducting silver paint, allowed to air-dry for 10–20 min, and sputter-coated with gold. Scanning electron micrographs were generated on a Hitachi S/570 microscope.

Bright-field microscopy

Dissected appendages from adult Drosophila were mounted in Canada balsam/methyl salicylate (6 g/4 ml) and viewed for light microscopy.

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