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Induction of *Drosophila* eye development by Decapentaplegic

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SUMMARY

The *Drosophila decapentaplegic (dpp)* gene, encoding a secreted protein of the transforming growth factor-β (TGF-β) superfamily, controls proliferation and patterning in diverse tissues, including the eye imaginal disc. Pattern formation in this tissue is initiated at the posterior edge and moves anteriorly as a wave; the front of this wave is called the morphogenetic furrow (MF). Dpp is required for proliferation and initiation of pattern formation at the posterior edge of the eye disc. It has also been suggested that Dpp is the principal mediator of Hedgehog function in driving progression of the MF across the disc. In this paper, ectopic Dpp expression is shown to be sufficient to induce a duplicated eye disc with normal shape, MF progression, neuronal cluster formation and direction of axon outgrowth. Induction of ectopic eye development occurs preferentially along the anterior margin of the eye disc. Ectopic Dpp clones situated away from the margins induce neither proliferation nor patterning. The Dpp signalling pathway is shown to be under tight transcriptional and post-transcriptional control within different spatial domains in the developing eye disc. In addition, Dpp positively controls its own expression and suppresses *wingless* transcription. In contrast to the wing disc, Dpp does not appear to be the principal mediator of Hedgehog function in the eye.

Key words: *decapentaplegic*, TGF-β, *hedgehog*, *Drosophila*, eye development, morphogenetic furrow, pattern formation

INTRODUCTION

Secreted molecules of the TGF-β superfamily play central roles in the development of both vertebrate and invertebrate organisms, controlling diverse processes such as the establishment of the body axes, cell proliferation and death, cell fate determination and differentiation (Kingsley, 1994; Hogan, 1996). The Drosophila TGF-β homologue *decapentaplegic (dpp)* is required throughout embryonic and larval development, controlling the establishment of dorso-ventral polarity and midgut formation in the embryo, and proliferation and patterning in the larval imaginal discs, the primordia of adult tissues. Studies of wing disc development have led to the view that Dpp exerts its influence over the entire disc. In this tissue, *dpp* expression is under the control of Hedgehog (Hh), a secreted factor acting as a short-range inducer of *dpp*. Ectopic Dpp induces the same range of effects as seen with ectopic Hh clones, leading to the view that in this tissue the primary function of Hh is to control the precise spatial domain of Dpp expression (Zecca et al., 1995).

Although Dpp has been shown to function in eye development, its precise role is not well understood. The compound eye of *Drosophila melanogaster* comprises an array of some 800 simple eyes called ommatidia. It is derived from the eye-antennal disc, which also contains the primordia of the antenna, the dorsal ocelli and surrounding head cuticle. The formation of the ommatidial array starts early in the third and final stage of larval life. A groove, called the morphogenetic furrow (MF), appears at the posterior edge of the disc and then sweeps anteriorly, leaving in its wake differentiating neurons (Fig. 1A). In the MF, cells arrest in G_1, change shape, assemble into clusters and begin to express a number of molecules associated with neuronal determination and differentiation (Heberlein and Moses, 1995).

Dpp plays an essential role in controlling proliferation and patterning in the developing eye disc. *dpp* is expressed along the posterior and lateral margins of the second instar disc long before ommatidia form (Blackman et al., 1991). Upon the onset of ommatidial differentiation in early third instar, *dpp* expression is extinguished at the posterior edge and becomes localized to the advancing MF (Fig. 1A) (Blackman et al., 1991). Several lines of evidence indicate that Dpp is involved in initiation of the MF. The hypomorphic eye-specific allele $dpp^{d\text{-}blk}$ blocks MF initiation along the lateral margins of the eye disc (Treisman and Rubin, 1995), and mutations in *Mothers against dpp (Mad)*, a downstream effector of Dpp signaling, block MF initiation anywhere along the posterior or lateral margins (Newfeld et al., 1996; Wiersdorff et al., 1996). In addition, Wingless (Wg), a Wnt-type growth factor, has been shown to antagonize Dpp activity, preventing MF initiation from the dorsal and ventral edges of the eye disc (Treisman and Rubin, 1995).

The role of Dpp in MF propagation is controversial. In the eye disc, Hh is secreted by differentiating neurons posterior to the MF, and it drives *dpp* expression and MF propagation across the disc (Heberlein et al., 1993; Ma et al., 1993). Patched (Ptc) and Protein Kinase A (PKa-C1), instead, act as negative regulators of *dpp* expression and eye development ahead of the MF. Arrest of MF progression in hh^{1} or hh^{ts2} mutant discs is

associated with a severe reduction or elimination of *dpp* expression (Heberlein et al., 1993; Ma et al., 1993). Conversely, patches of tissue producing Hh, or lacking either Ptc or PKa-C1 activity, induce Dpp, MF formation and ommatidial differentiation anterior to the normal MF (Heberlein et al., 1995; Strutt et al., 1995; Pan and Rubin, 1995). Recent studies on the role of *Mad* have led to the view that Hh function in MF progression may not be mediated by Dpp (Wiersdorff et al., 1996). However, a requirement for lower levels of Dpp signalling in progression cannot be excluded since the *Mad* alleles used retained some activity. Indeed, recent studies by Chanut and Heberlein (personal communication) support a role for Dpp in MF propagation.

The study of Dpp function in the eye and other discs through loss-of-function analysis has been impeded by haploinsufficiency of *dpp*, its involvement in proliferation and its cell non-autonomous properties. Through ectopic expression studies, however, Dpp's influence on growth and patterning in other discs has been uncovered (Capdevilla and Guerrero, 1994; Zecca et al., 1995; Nellen et al., 1996; Lecuit et al., 1996). In this paper we describe the effect of ectopic expression of Dpp in the eye-antennal disc. Dpp is sufficient to induce initiation of ectopic MFs and formation of duplicated eye discs selectively along the margins. Whereas eye tissue away from the margins can respond to Hh, it is not competent to respond to ectopic Dpp. These studies reveal that precise spatial control of Dpp expression and responsiveness to it are important determinants regulating patterning in the eye imaginal disc.

MATERIALS AND METHODS

Fly stocks

Flies carrying the *Actin>CD2>Gal4* and *UAS:hh* transgenes were generated by P-element transformation. The *Actin>CD2>Gal4* line used showed hsp70-Flip-dependent *lacZ* expression from a *UAS:lacZ* transgene. The *UAS:lacZ* (A. H. Brand), *UAS:en* (N. Perrimon) and *UAS:dpp* (K. Staehling-Hampton) transgenic lines and the Gal4 enhancer-trap lines *patched*-559.1 and 30A were provided by the Bloomington Drosophila Stock Center; the E132 Gal4 line by W. Gehring; the *tubulin>CD2, y+>dpp* used in the initial flip-out experiments and the *y hsp70-flp* stock by G. Struhl; the *dpp-lacZ* reporter H1.1 is described in Blackman et al. (1991); the *wg-lacZ* line is described in Kassis et al. (1992).

Dpp and Hh expression under the control of Gal4 lines

Males carrying the *UAS:dpp* or the *UAS:hh* transgene were mated to females of the Gal4 lines and their progeny was stained for the neuronal marker Elav (anti-Elav mAb from G. Rubin). Out of 56 *E132; UAS:dpp* discs, 41 discs showed ectopic MFs at the ventral or anterior margins, six discs showed growth only and nine discs were very small in size and completely filled with neuronal clusters. Most of these phenotypes were also seen in experiments generating random Dpp clones using the Gal4/Flip-out system. Out of 43 *E132; UAS:hh* discs, 20 discs showed ectopic ommatidial differentiation and growth ahead of the normal furrow; the remaining 23 discs were normal. Additional crosses were carried out with *wg-lacZ* or *dpp-lacZ* in the background to detect expression of these genes by X-gal staining; changes in gene expression were confirmed by in situ hybridization.

Generation of random Dpp-expressing clones

Females with the genotype *hsp70-flp; UAS:dpp; +* were mated to *Actin>CD2>Gal4; +; +* males: (1) without any other reporter constructs; (2) carrying *UAS:lacZ*; (3) carrying *dpp-lacZ*; (4) carrying *wg-lacZ*; or (5) carrying *UAS:en* or *UAS:fasII*. After 2 days of egg laying, adults were removed and the progeny were aged for 2 more days, heat shocked (34˚C for 30 minutes), and further incubated at RT for 1 to 2 days prior to dissection. Dissections and staining were carried out until the 10th day post-egg laying. The frequency of ectopic MFs or overgrowth increased from <10% of the discs on the first day of dissections to >70% 4 days later. Experiments with other *UAS:cDNA* constructs (e.g.*UAS:lacZ* and *UAS:en*) did not initiate ectopic MFs under the same conditions. Wing discs from *Actin>CD2>Gal4 /hsp70-flp;* UAS:*dpp/+; +/+* larvae displayed size and shape abnormalities consistent with ectopic expression of Dpp in this tissue (Capdevilla and Guerrero, 1994; Nellen et al., 1996; Lecuit et al., 1996). Duplications of leg and antennal discs were also observed.

To mark the location of the random Dpp-expressing clones, crosses were carried out as described above with a *UAS:lacZ* construct in the background. In 65 out of 72 cases with ectopic MF (57/72) or growth at the anterior disc margin (8/72), β-gal-marked clones were present in the eye-antennal disc. In the remaining seven discs, however, no clones were detected. Due to the use of paraformaldehyde in the first fixation (see Histology), it is likely that not all clones present in these eye-antennal discs were detected. In addition, some clones may die prior to the mid-third instar stage subsequent to inducing endogenous *dpp* expression.

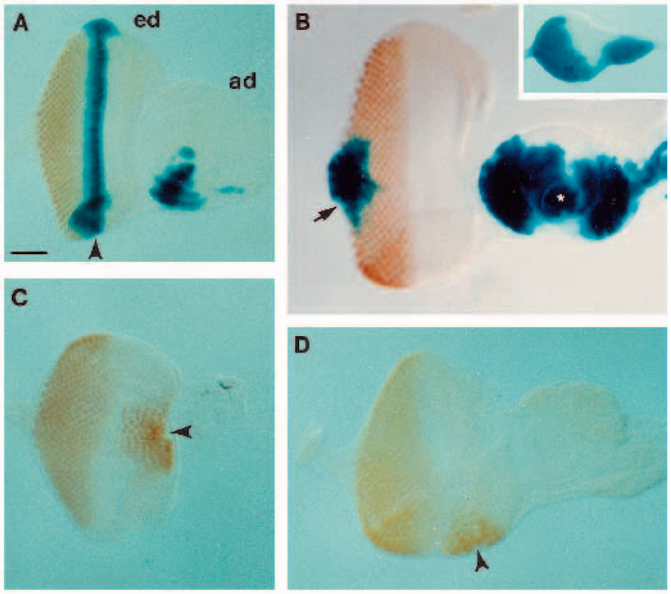

Fig. 1. Ectopic expression of *dpp* and induction of ectopic MFs. In all panels, eye-antennal discs are oriented with posterior to the left, anterior to the right and ventral down, unless otherwise indicated. (A) Wild-type third instar eye disc stained for Elav (brown) to visualize the developing ommatidia and for the *dpp-lacZ* reporter H1.1 (blue) to detect expression of the *dpp* gene in the MF (arrowhead) (ed, eye disc; ad, antennal disc). (B) Expression pattern driven by the Gal4 enhancer-trap line E132. Third instar *Gal4 E132; UAS:lacZ* eye-antennal disc stained for Elav to visualize the ommatidial array and for β-gal to detect expression of Gal4. Expression is present in the antennal disc (white asterisk) and at the very posterior edge of the eye disc (arrow). Inset: second instar *Gal4 E132; UAS:lacZ* eye-antennal disc stained for β-gal to detect expression of Gal4. (C,D) Elav-stained *Gal4 E132; UAS:dpp* eye-antennal discs with ectopic MFs. The ectopic MFs initiated at the anterior (C) and ventral (D) margins of the eye disc (arrowheads). Bar, 50 μm.

Histology

Expression of Elav, Dac and Eya was detected using mAbs provided by G. Rubin (anti-Elav mAb), G. Mardon (anti-Dac mAb 2-3) and S. Benzer (anti-Eya mAb). *dpp-lacZ* and *wg-lacZ* expression was detected by X-gal staining of discs fixed in PBS-0.8% gluteraldehyde. To detect both Elav and β-gal expression (*UAS:lacZ*, *dpp-lacZ* and *wg-lacZ*) eye-antennal discs were fixed in 2% paraformaldehyde (5 minutes), stained for β-gal activity (X-gal) at 37˚C (4-10 hours), incubated in 2% paraformaldehyde (30 minutes) and stained for the neuronal antigen Elav. Direct detection of gene expression (*dpp* and *wg* in *E132;UAS:dpp* discs; *eyeless* and *string* in disc with random *dpp*-expression clones) was carried out by situ hybridization with digoxygenin-labelled RNA probes. cDNA clones were provided by R. Blackman, R. Nusse and W. Gehring. To visualize the proliferation pattern in discs with random *dpp*-expressing clones, third-instar eye-brain complexes were incubated in Schneider's medium with bromodeoxyuridine (BrdU, 80 μg/ml) for 50 minutes at room temperature, fixed and double-stained for BrdU (anti-BrdU mAb from Becton-Dickinson) and Elav. BrdU incorporation was generally reduced as compared to wild type, but restricted regions of higher proliferation occurred in many eye-antennal discs. The second waves of cell divisions associated with the normal and ectopic MFs were either both lacking (32 discs) or both present (10 discs).

RESULTS

Ectopic Dpp induces MF initiation in the eye disc

To assess whether Dpp is sufficient to induce ommatidial development, we expressed Dpp in specific domains in the eye-antennal disc using the Gal4/UAS method of transcriptional activation (Brand and Perrimon, 1993). Females carrying a *UAS:dpp* transgene were mated to males from four different Gal4 enhancer-trap lines. Two lines (patched-559.1 and sca-375.1) resulted in lethality prior to eye development, and were, therefore, uninformative. Line 30A drives expression in two lateral patches from early in the second instar. These regions of the disc normally express Dpp. However, eye development does not initiate at these sites due to the presence of suppressors of Dpp activity such as Wg (Treisman and Rubin, 1995; Ma and Moses, 1995). Increased expression of Dpp under 30A control was not sufficient to overcome these inhibitory effects as all discs looked indistinguishable from wild type. In contrast, ectopic MFs were induced by Dpp driven by the Gal4 enhancer-trap line E132.

In the second instar, E132 drives expression throughout much of the ventral region of the eye-antennal disc, with staining expanding posteriorly to cover the entire dorsoventral extent of the eye disc (Fig. 1B inset). In the third instar, expression is found in the antennal disc and in a small patch of cells at the posterior of the eye disc near the optic stalk (Fig. 1B). Since progeny carrying E132 and *UAS:dpp* did not survive to adulthood, we were restricted to analyzing effects in third instar larvae. In most discs, an ectopic MF initiated at the anterior edge of the eye disc and propagated posteriorly, i.e. in a direction opposite to

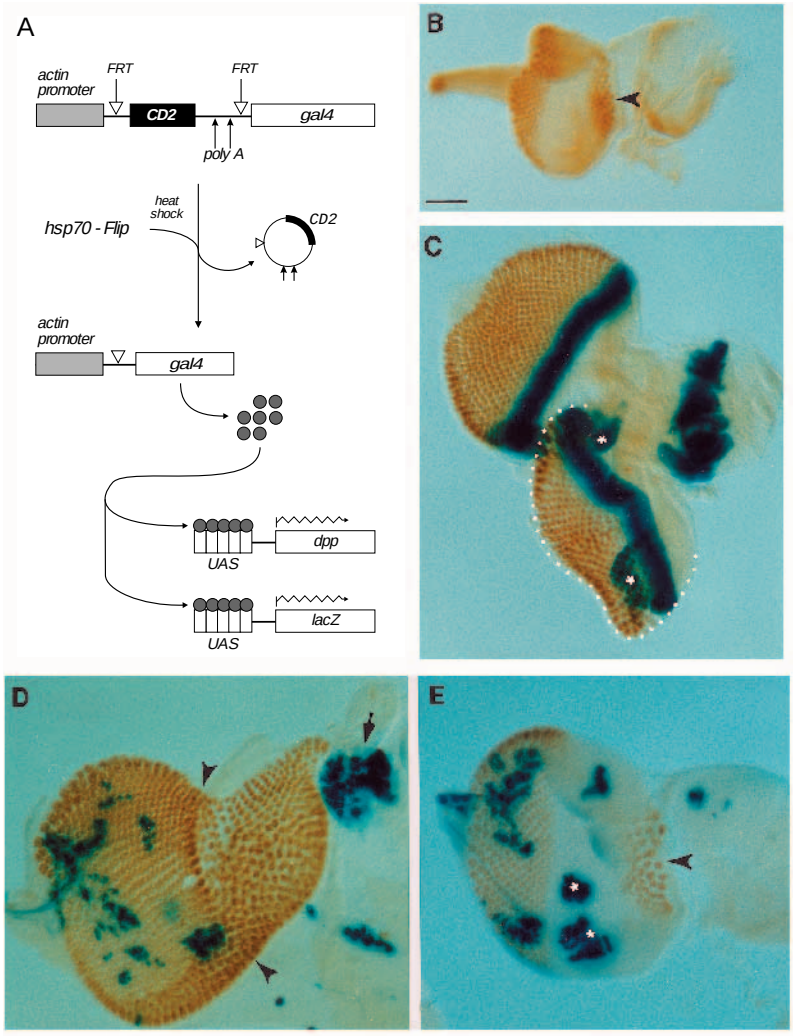

Fig. 2. Induction of ectopic MF and duplicate eye disc by random Dpp-expressing clones. In B, D and E, posterior (normal eye) is to the left and anterior (ectopic eye) is to the right. (A) Schematic diagram of the method used to generate ectopic Dpp clones. (B) Third instar eye discs stained for Elav; an ectopic MF can be seen propagating from the anterior margin of the disc (arrowhead) towards the posterior. Development of the ectopic eye is not associated with local overgrowth. (C) Third instar eye-antennal disc stained for Elav and *dpp-lacZ*. Proliferation has led to the formation of a duplicated eye disc. The new eye disc (white outline) is indistinguishable from a normal eye disc, but can be recognized by the absence of a connection to the brain. The lateral folds (white asterisks) are typically seen in normal discs. The new eye disc originates from the anterior edge of the normal eye disc where it connects to the antennal disc. The spatial relationship between discs is best established by careful observation under a dissection microscope prior to mounting; mounting often obscures the spatial relationships between the normal disc and regions of ectopic growth. Note that β-gal staining in this disc is due to the *dpp-lacZ* reporter and not to *UAS:lacZ*. (D,E) Third instar eye-antennal discs were stained for Elav to visualize developing ommatidia and for β-gal to detect clones producing ectopic Dpp from the *UAS: dpp* transgene. (D) A large clone (arrow) is present in the antennal disc next to the anterior margin of the eye disc. The ectopic (right) and normal (left) eye fields in this disc have already fused (arrowheads), forming a continuous lawn of neuronal clusters. (E) Several β-gal-marked clones (white asterisks) are present just ahead of the normal MF, but none are associated with ommatidial development; in addition, none of the clones is closely associated with the site of origin of the ectopic eye (arrowhead). Bar, 50 μm.

the normal MF (Fig. 1C). Overgrowth was often observed at this site. Less frequently, the ectopic MF initiated from the ventral margin and propagated dorsally (Fig. 1D).

Dpp induces ectopic MFs selectively at the anterior margin and acts at a distance

In order to further assess the regional specificity of MF induction, ectopic Dpp expression was induced in random patches. Dpp-expressing clones generated by the interruption cassette technique of Struhl and Basler (1993) did not lead to alterations in eye development and produced weak effects in the wing. Since the level of Dpp expression driven by this construct was rather low (Zecca et al., 1995; Nellen et al., 1996), the consequences of higher levels of Dpp were assessed. This was achieved using a hybrid of the Flip-out and Gal4 activation systems (Struhl and Basler, 1993; Brand and Perrimon, 1993). Clones expressing Gal4 were induced by 'flipping out' an interruption cassette from an *Actin>CD2>Gal4* transgene in a genetic background containing *UAS:dpp* (Fig. 2A). Due to late larval and pupal lethality, the effect of ectopic expression was studied in third instar larvae. Ectopic MFs that propagated posteriorly from the anterior margin were observed in many eye discs (Fig. 2B). Overgrowth of anterior eye disc tissue occurred frequently, and in some cases a new eye disc was generated (Fig. 2C). Thus, regardless of the pattern of expression, the response to ectopic Dpp was remarkably specific: only one ectopic MF was induced and it initiated consistently from the anterior margin of the eye disc around the dorsoventral midline.

The locations of the Dpp-expressing clones were mapped by co-expression of *UAS:lacZ* and *UAS:dpp* transgenes in cells that had lost the interruption cassette separating the *actin* promoter from *gal4* (Fig. 2A). Ectopic MFs were not always associated with β-gal expression at the site from which the ectopic MFs initiated (i.e. anterior border of the eye disc near the dorsoventral midline) (Fig. 2D and 2E). Moreover, although β-gal-labeled clones were found just anterior to the MF (i.e. in a region competent to induce MFs in response to ectopic Hh or loss of Ptc or PKA-C1 activity), eye development was never initiated at these sites (Fig. 2E). 20 eye discs had one or more lacZ-marked ectopic Dpp clones located just ahead of the normal MF. None of these clones showed ectopic development, as assessed by Elav staining or overgrowth. These results not only confirmed that specific regions of the eye disc are competent to respond to Dpp, but also indicated that ectopic Dpp exercised its effect at a considerable distance. Recently, evidence that Dpp can act directly at some distance in the wing disc has been presented (Nellen et al., 1996; Lecuit et al., 1996).

Development of the ectopic ommatidial array is similar to wild type

The genes *eyes absent (eya), dachshund (dac) and sine oculis (so)* are required early in eye development and are normally expressed in the eye disc as early as mid-to-late second instar, prior to ommatidial differentiation (Bonini et al., 1993; Mardon et al., 1994; Cheyette et al., 1994; F. Pignoni, unpublished). Increased levels of the So, Eya and Dac proteins were detected at the anterior edge of the eye disc prior to MF movement (Fig. 3A,B; Eya showed a pattern identical to So, not shown). The *eyeless* (*ey*) gene plays a major role in eye development and can induce formation of ectopic eyes in tissues other than the eye disc (Halder et al., 1995). In wild type, *ey* expression is restricted to the region anterior to the MF (Quiring et al., 1994). In discs with ectopic Dpp clones, the *ey* transcript was clearly detected throughout the region of overgrowth prior to MF movement (Fig. 3C). Thus, the eye disc tissue initiating the ectopic MF falls within the *ey* expression domain. In discs with a propagating ectopic MF, *ey* expression was restricted to the region between the two advancing MFs (data not shown). Induction of the cell cycle regulator *string* was also observed before initiation (Fig. 3D) and ahead of the propagating ectopic MF. Although the proliferative response to ectopic Dpp expression was complex (see Materials and Methods), synchronization of cell cycle progression in the ectopic MF could be observed in many discs (Fig. 3E). The endogenous *dpp* gene was also induced early and its expression was present in the propagating MFs (Figs 2C, 4A and 5). Ectopic expression of *dac*, *dpp* and *stg*, but not *ey, eya* and *so*, also was detected in the antennal disc (data not shown). Induction of later markers, such as the neuronal antigens Elav and 22C10, was largely normal (Figs 1, 2B-E, 3F and 4A-B). Most clusters stained with mAb Elav were indistinguishable from wild type. However, abnormal clusters containing variable numbers of neurons were seen at the initiating site of the ectopic eye field. Finally, photoreceptor cell axons projected toward the site of initiation of the new eye field and converged onto a central point (Fig. 3F). In a few cases, the axons from the ectopic eye field appeared to generate a structure similar to an optic stalk, which, however, did not connect to the brain.

Dpp suppresses *wg* expression

In some discs, the ommatidial array extended into regions of the disc that normally give rise to the head cuticle surrounding the compound eye. In these discs, the normal and ectopic eye fields fused, forming a lawn of neuronal clusters covering essentially the entire disc (Fig. 4A,B). Since Wg is required for the development of head cuticle and is expressed along the dorsal and ventral sides of the third instar eye disc (Fig. 4C) (Treisman and Rubin, 1995), we assessed *wg* expression in these discs. Wg expression was detected by staining for a *wg-lacZ* enhancer-trap line that reflects the expression of the *wg* gene (Kassis et al., 1992) and by in situ hybridization with a *wg* RNA probe. A marked decrease in Wg expression along both margins was observed in many discs with ectopic MFs (Fig. 4D). Moreover, in young *E132; UAS:dpp* discs, loss of ventral *wg* expression was detected prior to ommatidial differentiation (data not shown). Thus, increased Dpp levels caused by expression in the advancing MFs (in discs with Dpp clones) or along the ventral margin prior to initiation (in *E132; UAS:dpp* discs) suppress *wg* expression.

Endogenous *dpp* is induced early along the margins but not in the center of the eye disc

The earliest change induced at the anterior margin by Dpp-expressing clones was activation of endogenous *dpp* expression in second instar discs (Fig. 5A). *dpp* expression was detected by staining for a *dpp-lacZ* reporter construct that reflects the expression of the *dpp* gene (Blackman et al., 1991), and was confirmed by in situ hybridization. Expression at the anterior margin appeared to be higher than at the posterior, extended

along the entire anterior margin and was, in many cases, associated with tissue overgrowth (Fig. 5A,B). In most discs, the anterior domain of Dpp expression stopped just short of the posterior Dpp domain. The lateral regions lacking Dpp correspond to areas of high Wg expression, suggesting that Wg may play a role in setting the boundaries of *dpp* expression. Similarly, a requirement for Wg in restricting the normal *dpp* domain laterally is shown by the ectopic activation of *dpp* along the anterior margin of *wg* mutant discs at the non-permissive temperature (Ma and Moses, 1995). Initiation of the new MF always occurred close to the midline (i.e. in the center of this Dpp domain) and then spread laterally in both directions (Fig. 5C). In *E132; UAS:dpp* discs, the anterior domain of Dpp expression often merged with the posterior domain on the ventral side (Fig. 5D). Activation of endogenous *dpp* ventrally is most likely due to the expression of exogenous Dpp on the ventral side of second instar discs under the control of E132 Gal4. This expression pattern is consistent with the observed suppression of *wg* expression and initiation of ectopic MFs from the ventral side (Fig. 1D).

Neither induction of the endogenous *dpp* gene nor ommatidial differentiation were ever observed in the center of the eye disc (n>350 discs with random Dpp clones). The lack of *dpp* induction most likely reflects differences in the Dpp signalling pathway in the center of the disc as opposed to the margins. Alternatively, ectopic Dpp may not be produced in an active form within this region of the disc. However, since Dpp acts at a distance, this possibility does not explain why increased Dpp production along the margins does not result in activation of endogenous *dpp* in more central regions.

The response to ectopic Dpp and Hh differs significantly

Since Hh is known to induce MF initiation in clones located just anterior to the normal MF but not further anteriorly, it appears that eye disc tissue responds differently to ectopic Hh and Dpp. Whereas E132 Gal4-controlled expression of Dpp induced ectopic MFs initiating exclusively from the edges, E132 Gal4-induced expression of Hh led to massive overgrowth and induction of eye development throughout most of the region anterior to the MF. No obvious directionality to ommatidial recruitment could be seen, most likely reflecting the initiation of multiple MFs (Fig. 6A,B). Induction of the *dpp-lacZ* reporter in *E132; UAS:hh* discs extended into the central region of both second and third instar eye discs (Fig. 6C,D). Thus, initiation of ectopic MFs exclusively from the margins of the eye disc is a response specific to ectopic Dpp expression and reflects differences in the tissue responsiveness to Dpp and Hh.

DISCUSSION

In this paper we demonstrate that ectopic expression of Dpp is sufficient to induce eye disc duplications along the anterior margin of the eye disc. The lack of direct correspondence between the location of the clones and the ectopic eyes suggests that Dpp can act at a distance. Since proliferation per se is not sufficient to generate an eye disc (i.e. duplicated discs are not induced by ectopic Hh, although cells are induced to proliferate and differentiate), ectopic Dpp must coordinate growth and differentiation in a way that fully recapitulates the entire wild-type developmental program.

The generation of the duplicated disc may result from the pattern of endogenous *dpp* induced and the positioning of MF initiation within this domain (Fig. 5A-C). Expression of endogenous *dpp* was induced by exogenous Dpp in a broad domain along the anterior margin, resembling its expression at the posterior edge of the normal disc. This is consistent with recently published evidence that Dpp positively regulates its own expression in the eye disc. Expression of *dpp* is dependent on both the signalling cascade (i.e. Dpp is absent in *Mad* mutant clones located along the posterior margin of the disc; Wiersdorff et al., 1996) and the signal itself (i.e. Dpp is reduced in $dpp^{d\text{-}blk}$ mutant discs and its expression in the MF decreases after shifting temperature-sensitive *dpp* mutant larvae to the non-permissive temperature; Wiersdorff et al., 1996; Chanut and Heberlein, personal communication). Initiation of the MF always occurred in the center of the ectopic *dpp* domain and spread laterally. This pattern of initiation was seen in all ectopic MFs induced and may reflect the control of Dpp activity by suppressors such as Wg present at lateral positions within the eye disc (Treisman and Rubin, 1995). Interestingly, initiation of eye development at either the ectopic or the normal site occurred only in third instar, although Dpp was expressed at these sites in second instar larvae. Hence, the factors controlling the temporal specificity of MF initiation at the posterior (normal MF) and the anterior (ectopic MF) are similar.

Based on these observations, we favor a model (Fig. 7) in which ectopic Dpp activates the eye development program by inducing expression of the endogenous *dpp* gene at the anterior margin of the eye disc at an early stage. The endogenous Dpp then initiates an autoregulatory loop at the anterior edge that ensures lateral spreading and maintenance of *dpp* expression independently of the original Dpp source. During second instar, this Dpp domain induces the appropriate pattern of proliferation in the surrounding tissue and, in the third instar, it initiates eye development at the anterior site at a similar time to initiation at the posterior edge. Once initiated, the developing ommatidial array generates its own internal organizational cues, forming a second, normal-looking eye disc (Chanut and Heberlein, 1995). In some cases, an MF initiates at the anterior edge without giving rise to a disc-like structure. This appears to be the effect of clones induced later in development, too late for sufficient proliferation to occur prior to MF initiation.

The changes induced by ectopic Dpp at the anterior margin may be viewed as a re-specification of anterior margin into posterior. Thus, the anterior margin would acquire, along with the transcriptional program, the organizing functions of the posterior margin. What these organizing functions consist of is an issue that needs further attention. The Dpp domain may simply serve an early proliferative role and later act in MF initiation and propagation. Alternatively, Dpp may play an additional role at an early stage in specifying an eye primordium, as distinct from the other tissues derived from the eye disc. This early function of Dpp would be analogous to the role of the anterior-posterior system in specifying the wing primordium as distinct from the body wall in the second instar wing imaginal disc (Ng et al., 1996).

The effects of ectopic Dpp underscore the complex regula-

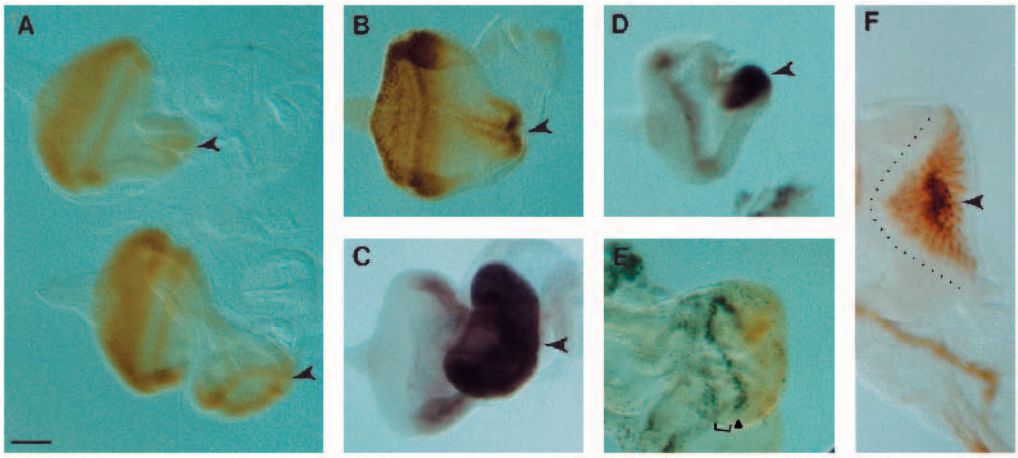

Fig. 3. The development of the ectopic ommatidial array is similar to wild type. In all panels posterior (normal eye) is to the left and anterior (ectopic eye) is to the right. (A) Induction of So expression (detected using an antibody) at the anterior margin of the eye discs (arrowheads) prior to MF initiation (upper disc), and associated with the ectopic MF during progression (lower disc). (B) Higher levels of Dac protein (detected using mAb Dac 2-3) at the initiation site of ectopic MF (arrowhead) prior to MF progression. (C) In situ hybridization with a digoxygenin-labelled *ey* probe. Strong expression is detected throughout the region of overgrowth (arrowhead) prior to ectopic MF initiation. (D) Early induction of *stg* transcription at initiation site of ectopic eye (arrowhead) detected by in situ hybridization with a digoxygenin-labelled cDNA probe. (E) Arrest of cell proliferation (bracket) and second wave of cell divisions (arrowhead) in the ectopic eye disc, as detected by BrdU incorporation. (F) Staining of axonal projections with mAb 22C10; neurons project their axons away from the MF (approximate position shown by dotted line) and converge towards a central point along the anterior edge (arrowhead). Bar, 50 μm (A-E); 100 μm (F).

tion of Dpp expression and activity in the eye disc. In addition to the posterior margin, where *dpp* is already expressed in very young second instar larvae, the eye disc can be subdivided into three different regions based on regulation of *dpp* expression and competence to respond to the Dpp signal. Away from the margins, the Dpp signalling pathway is under strict transcriptional and post-transcriptional regulation. Dpp-expressing clones located in the middle of the eye disc (i.e. in a region competent to induce *dpp* and MFs in response to ectopic Hh, or loss of Ptc or PKA-C1 activity) neither induced ectopic MFs nor endogenous *dpp*. Whereas *ptc* and *pka-C1* suppress *dpp* expression ahead of the MF (Ma and Moses, 1995; Pan and Rubin, 1995; Strutt et al., 1995), the genes antagonizing Dpp activity away from the margins are not known. The failure of Dpp to induce MFs away from the margins also indicates that, while Dpp may still be required for MF progression, it is not sufficient. Presumably, other factors induced by Hh are necessary to render the tissue ahead of the MF responsive to Dpp.

Dpp is differentially regulated along the lateral and anterior margins. The lateral margins of the wild-type eye disc already express Dpp but do not respond to the Dpp signal by initiating MFs. Dpp activity is suppressed post-transcriptionally by Wg dorsally and by Wg and other factors ventrally, since ommatidial development can occur at these sites upon loss of Wg activity (Treisman and Rubin, 1995; Ma and Moses, 1995). The occurrence of MFs initiating from the ventral margin, in some of our experiments, indicates that the ventral margin of the eye disc is, indeed, competent to respond to the Dpp signal when this signal is increased in an otherwise wild-type disc. These MFs were observed in the *E132; UAS:dpp* experiment, but never when *UAS:dpp* expression was driven by the weaker lateral-margins-specific line A30, or in randomly distributed clones. These results indicate that the post-transcriptional suppression of Dpp activity along the lateral margins can be overcome by high levels of Dpp in these regions.

The anterior margin, while not normally expressing *dpp*, is competent to express *dpp* upon induction and can respond to the Dpp signal. In a wild-type disc, therefore, *dpp* transcription must be repressed at this site to prevent formation of a second MF or eye disc. The factor(s) involved in the control of *dpp* at the transcriptional level in this region of the disc are not known, but they include Wg, since lateral suppression of Dpp activity by Wg is required (Treisman et al., 1995; Ma and Moses, 1995). Interestingly, when an endogenous *dpp* domain

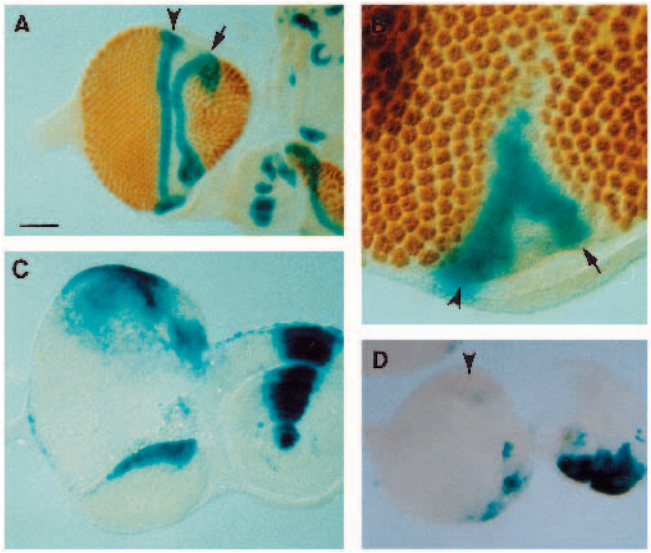

Fig. 4. Repression of *wg* expression by Dpp. (A,B,D) Discs with random ectopic Dpp clones. (A,B) Third instar discs double-stained for *dpp-lacZ* (blue) and Elav (brown). Once the normal MF (arrowhead) and the ectopic MF (arrow) merge in the middle of the disc, expression of the *dpp-lacZ* reporter H1.1 disappears, leaving a disc filled with ommatidia. (C) *wg-lacZ* expression along the dorsal and ventral margins of a wild-type eye disc. (D) In discs with ectopic growth or ectopic clusters, invariably associated with high levels of Dpp, loss of *wg-lacZ* expression is most pronounced along the ventral margin (arrowhead). Note that in D ventral is up, whereas in C ventral is down. Bar, 50 μm (A,C,D); 125 μm (B).

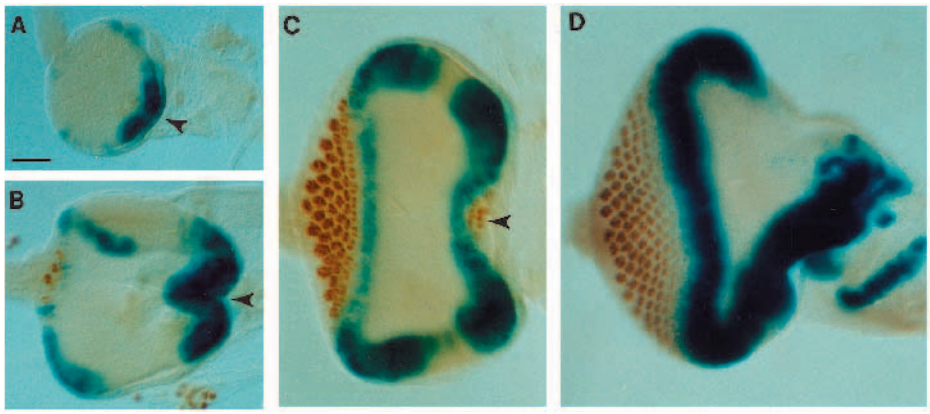

Fig. 5. Ectopic induction of endogenous *dpp* occurs early and is always restricted to the margins. Eye-antennal discs were stained for Elav (brown) to visualize differentiating ommatidia and for the *dpp-lacZ* reporter H1.1 (blue) to detect induction of endogenous *dpp* gene. (A) Induction of the *dpp-lacZ* reporter at the anterior margin of a young second instar disc. Expression at the anterior margin (arrowhead) can already be detected after 4 hours of X-gal staining; the normal expression along the posterior requires staining for more than 10 hours. (B) Expression of the *dpp-lacZ* reporter and associated overgrowth (arrowhead) along the anterior margin of a young third instar disc. (C) Initiation of the ectopic MF (arrowhead) always occurs at the center of the anterior *dpp* domain. (D) Expression of the *dpp-lacZ* reporter extends along the ventral and anterior margins in *Gal4 E132; UAS:dpp* discs. Bar, 100 μm.

is induced at the anterior margin by ectopic Dpp clones it does not extend around the entire margin, but stops laterally, abutting a region of high Wg expression. These observations suggest that the inhibition of Dpp activity by Wg may also play a role in defining the lateral extent of the Dpp expression domain in the wild-type eye disc. Wg may act by blocking a lateral spread of *dpp* by inhibiting Dpp autoregulation.

Not only does Wg regulate Dpp, Dpp may in turn regulates *wg*. In *E132; UAS:dpp* discs, ectopic Dpp expression along the ventral margin resulted in suppression of *wg* expression prior to ommatidial differentiation. Conversely, Wg expression is expanded in discs mutant for the hypomorphic dpp^{blk} allele (Wiersdorff et al., 1996). Thus, reciprocal regulation between Wg and Dpp may play an important role in the development of the eye disc as a whole, setting the balance of eye versus cuticle derivatives. This interaction is most similar to dorso-ventral patterning in the leg disc, where mutual repression of Dpp and Wg is essential to restrict the dorsalizing and ventralizing activities of these two molecules to opposite regions of the disc (Penton and Hoffman, 1996; Jiang and Struhl, 1996).

Dpp, Wg and Hh control proliferation and patterning in essentially all imaginal discs studied. However, their relationship to one another differs from tissue to tissue. In contrast to the developing wing, in the eye disc Dpp does not fall strictly under the control of Hh nor is it the principal mediator of Hh function. Whereas, the antagonistic relationship between Dpp and Wg in the eye disc is similar to that in the leg, it is different

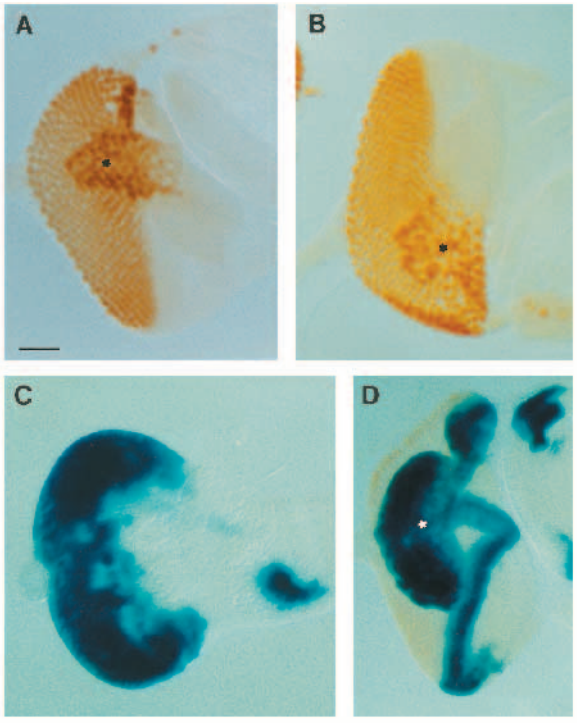

Fig. 6. Eye disc tissue away from the margins can respond to ectopic Hh. (A,B) Elav staining of *Gal4 E132; UAS:hh* eye-antennal discs. Ommatidial differentiation (asterisks) can be seen within an overgrown region in the eye disc. The regions of overgrowth lie below the normal disc epithelium. There is no evidence of an MF initiating from the anterior margin. (C) A *Gal4 E132; UAS:hh* second instar disc stained for *dpp-lacZ*. The region of induction of the endogenous *dpp* gene extends away from the margins towards the center of the disc. (D) A *Gal4 E132; UAS:hh* third instar disc stained for *dpp-lacZ*. A large overgrown region in the center of the disc expresses the reporter (white asterisk). Bar, 50 μm (A,B,D); 100 μm (C).

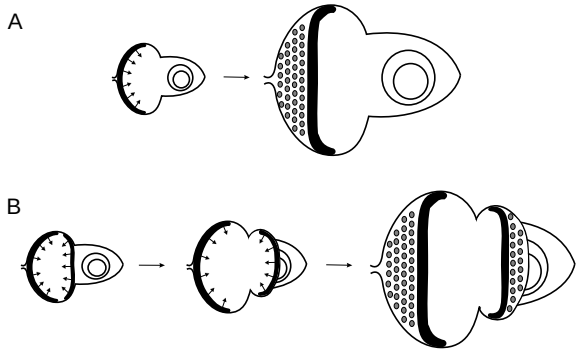

Fig. 7. Eye duplication. Dpp expression in the developing eye disc is represented by the thick line, differentiating ommatidial clusters by shaded circles. Arrows indicate Dpp activity in second instar discs. (A) In normal development, the Dpp domain at the posterior edge controls growth and patterning. (B) In discs expressing Dpp at the anterior edge, a second eye disc is formed.

from that in the wing disc. Since these imaginal discs give rise to very different adult structures, additional factors determine the specificity of the response to these extracellular signals. Genes such as *eyeless, sine oculis, eyes absent and dachshund* are likely to play important roles in determining the specificity in the eye disc (Halder et al., 1995; Cheyette et al., 1994; Bonini et al., 1993; Mardon et al., 1994).

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