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Dpp signaling thresholds in the dorsal ectoderm of the *Drosophila* embryo

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SUMMARY

The dorsal ectoderm of the *Drosophila* embryo is subdivided into different cell types by an activity gradient of two TGFβ signaling molecules, Decapentaplegic (Dpp) and Screw (Scw). Patterning responses to this gradient depend on a secreted inhibitor, Short gastrulation (Sog) and a newly identified transcriptional repressor, Brinker (Brk), which are expressed in neurogenic regions that abut the dorsal ectoderm. Here we examine the expression of a number of Dpp target genes in transgenic embryos that contain ectopic stripes of Dpp, Sog and Brk expression. These studies suggest that the Dpp/Scw activity gradient directly specifies at least three distinct thresholds of gene expression in the dorsal ectoderm of gastrulating embryos. Brk was found to repress two target genes, *tailup* and *pannier*, that exhibit different limits of expression within the dorsal ectoderm. These results suggest that the Sog inhibitor and Brk repressor work in concert to establish sharp dorsolateral limits of gene expression. We also present evidence that the activation of Dpp/Scw target genes depends on the *Drosophila* homolog of the CBP histone acetyltransferase.

Key words: Dpp thresholds, Brinker, CBP, Sog, *Drosophila melanogaster*

INTRODUCTION

The maternal Dorsal (Dl) gradient initiates the differentiation of the mesoderm, neurogenic ectoderm and dorsal ectoderm in the precellular *Drosophila* embryo. Dl establishes these tissues through the differential regulation of various target genes, including *snail* (mesoderm), *rho* (neurogenic ectoderm) and *dpp* (dorsal ectoderm) (reviewed by Mannervik et al., 1999). Subsequent signaling interactions subdivide each tissue into multiple cell types during gastrulation. For example, Dpp secreted from the dorsal ectoderm induces the underlying mesoderm to form the heart and gut muscles. In contrast, noninduced mesoderm gives rise to somatic muscles (Staehling-Hampton et al., 1994; Frasch, 1995). The neurogenic ectoderm forms the ventral epidermis and CNS, including three distinct rows of neurons in the ventral nerve cord and a specialized row of mesectodermal cells at the ventral midline (Weiss et al., 1998; McDonald et al., 1998). The dorsal ectoderm is subdivided into at least two tissues, the amnioserosa and dorsal epidermis (reviewed by Rusch and Levine, 1996; Podos and Ferguson, 1999).

This subdivision of the dorsal ectoderm requires a Dpp activity gradient in the dorsal half of the precellular embryo (Ferguson and Anderson, 1992; Wharton et al., 1993). The gradient is dependent on the activity of a second TGFβ signaling molecule, Screw, which potentiates Dpp signaling. Both Scw and Dpp are required to achieve a peak signaling threshold in the presumptive amnioserosa (Arora et al., 1994). Scw activity is modulated by the secreted Sog protein (Neul and Ferguson, 1998; Nguyen et al., 1998), which is related to the Chordin inhibitor of *Xenopus* (Holley et al., 1995). Sog is expressed in broad lateral stripes in the presumptive neurogenic ectoderm (François et al., 1994), possibly in direct response to the Dl gradient. This localized source of Sog is thought to inhibit and enhance Dpp/Scw signaling (reviewed by Podos and Ferguson, 1999). Peak levels of Sog within the lateral stripes completely block Dpp/Scw activity to permit neurogenesis. Intermediate levels of Sog secreted into dorsolateral regions attenuate Dpp/Scw signaling to specify the dorsal epidermis (Biehs et al., 1996; Marqués et al., 1997). Finally, low levels of Sog secreted into the dorsalmost regions somehow enhance Dpp/Scw signaling to give a peak threshold response and formation of the amnioserosa (Ashe and Levine, 1999).

A number of marker genes have been shown to exhibit distinctive patterns of expression in response to the Dpp/Scw activity gradient. For example, *Race* is expressed in the dorsalmost regions in response to peak Dpp-Scw activity (Rusch and Levine, 1997). In contrast, *zerknüllt* (*zen*) (Rushlow et al., 1987) and *rho* (Bier et al., 1990) appear to be expressed in somewhat broader patterns that span the amnioserosa and extend into the presumptive dorsal epidermis in dorsolateral regions. Finally, the GATA-related gene *pnr* (Ramain et al., 1993; Winick et al., 1993) is activated throughout the dorsal ectoderm; the lateral limits of the pattern appear to abut the lateral stripes of *sog* expression within the neurogenic ectoderm (Winick et al., 1993; Heitzler et al., 1996).

A putative transcriptional repressor, Brk, might help establish different Dpp signaling thresholds (Campbell and Tomlinson, 1999; Jazwinska et al., 1999a; Minami et al., 1999). Brk is expressed in lateral stripes in the neurogenic ectoderm in early embryos. These stripes are established by the maternal Dl gradient, and subsequently maintained by Dpp signaling, which suppresses *brk* transcription in the dorsal ectoderm. Thus far, just one putative Dpp/Scw target gene, *pnr*, has been shown to be repressed by Brk (Jazwinska et al., 1999b). It is therefore unclear whether Brk is required to yield multiple patterning thresholds in response to Dpp signaling.

In the present study, *dpp*, *brk* and *sog* were misexpressed in transgenic embryos using the *eve* stripe 2 enhancer (Kosman and Small, 1997). A number of target genes were analyzed, including *Race*, *rho*, *pnr* and members of the 'ush-group' of genes required for amnioserosa maintenance (Frank and Rushlow, 1996), such as *hindsight* (*hnt*) (Yip et al., 1997), *u-shaped* (*ush*) (Cubbada et al., 1997) and *tailup* (*tup*) (Thor and Thomas, 1997). The stripe2-*brk* transgene was found to repress *pnr* and *tup*, which are activated by low and intermediate levels of the Dpp/Scw activity gradient, respectively. The lateral limits of the *pnr* expression pattern are established by high levels of the Brk repressor, whereas the *tup* pattern appears to depend on lower levels of Brk. Evidence is also presented that the *Drosophila* homolog of the CBP histone acetyltransferase is specifically required for the activation of Dpp/Scw target genes at the onset of gastrulation.

MATERIALS AND METHODS

Drosophila stocks

The fly stocks used were as follows: *brinker*, *yw brk^{M68}*/FM7c *ftz-lacZ* (Jazwinska et al., 1999a); *decapentaplegic*, *dpp^{Hin37}*/GlaDp(2;2)DTD48; *gastrulation defective*, *gd^{7}*/FM3; *nejire*, *nej^{1}* FRT/FM7c (Akimura et al., 1997b). *nej^{1}* germline clones were induced using the Flp-DFS technique (Chou and Perrimon, 1996). Briefly, *nej^{1}* FRT/FM7c females were mated with *ovoD1* FRT; hs-FLP males. Larvae were heat shocked for 3 hours at 37°C on days 3, 4 and 5 after egg laying to induce expression of the FLP recombinase. Females lacking the Bar mutation present on the balancer chromosome were mated with *yw* males, and embryos were collected and fixed for in situ hybridization.

The misexpression constructs contain a FRT-transcription stop-FRT cassette to circumvent lethality (Kosman and Small, 1997) and were activated using a transgenic line homozygous for a P[*ry^{+}β$_2$-tubulin-flp*] insertion as described previously (Ashe and Levine, 1999). After removal of the *flp*-out cassette, it was found that expression of the stripe2-*brk*, stripe2-*sog* and stripe2-*dpp* transgenes is not lethal. To introduce stripe2-*brk* and stripe2-*dpp* into the same embryo, females containing the stripe2-*dpp* transgene were crossed to males carrying the stripe2-*brk* transgene.

Plasmid construction, P-element transformation and in situ hybridization

A *Dra*I-*Hin*dIII (465-2740) fragment of the *brk* cDNA and a *Nde*I-*Ssp*I (1190-3090) of the *dpp* cDNA from KS*dpp*Nde (Rusch and Levine, 1997) were blunt-end ligated into *Hin*dIII-*Not*I-digested SK+ which has *Asc*I sites engineered into the *Sac*II and *Sal*I sites of the polylinker. The *brk* and *dpp* cDNAs were then transferred as *Asc*I fragments into *Asc*I-digested 22FPE (Kosman and Small, 1997). P-element-mediated transformation and in situ hybridizations using digoxigenin-labeled RNA probes were performed as described (Jiang et al., 1991).

RESULTS

Different dorsal ectoderm genes were examined in a variety of mutant and transgenic embryos using digoxigenin-labeled RNA probes and in situ hybridization (Jiang et al., 1991). The normal expression patterns suggest the occurrence of at least three thresholds of gene activity in response to the Dpp/Scw activity gradient (Fig. 1). The *Race* and *hnt* genes are expressed in narrow strips in the dorsalmost regions of the embryo (Fig. 1A,B, respectively), although the anteroposterior limits of the two patterns are distinct. It is conceivable that early-acting segmentation genes are responsible for repressing *Race* in posterior regions and *hnt* in anterior regions. Somewhat broader expression patterns are observed for *tup* and *ush* (Fig. 1C,D). These patterns encompass the presumptive amnioserosa and dorsal regions of the dorsal epidermis.

Broad staining patterns are observed for two genes encoding GATA transcription factors, *dGATAc* (Lin et al., 1995) and *pnr* (Fig. 1E,F, respectively). *pnr* is expressed throughout the dorsal ectoderm in the presumptive thorax and abdomen (Fig. 1F). *dGATAc* exhibits a nearly reciprocal pattern in anterior and posterior regions; staining is mainly excluded from regions expressing *pnr*, although a weak patch of staining is detected in a portion of the presumptive amnioserosa. Most of the subsequent analyses on gradient thresholds focus on the regulation of *hnt* (Fig. 1B), *tup* (Fig. 1C) and *pnr* (Fig. 1F).

Manipulating *dpp* dose

All of the aforementioned genes are virtually silent in the dorsal ectoderm of *dpp^{-}*/*dpp^{-}* embryos (data not shown), while changes in *dpp^{+}* gene dose cause altered patterns of expression (Fig. 2). For example, increasing the number of *dpp^{+}* copies from two (Fig. 2A) to three (Fig. 2B) to four (Fig. 2C) results in a sequential expansion of the *hnt* expression pattern, whereas expression is lost in *dpp*/+ heterozygotes (data not shown). In contrast, *ush* is expressed in *dpp*/+ heterozygotes, although there is a marked narrowing in the expression pattern as compared with wild-type embryos (Fig. 2G, compare with H). Three copies of *dpp^{+}* cause an expansion of the *ush* pattern (Fig. 2I). Similarly, the *tup* expression pattern is narrower in *dpp*/+ heterozygotes and expanded in embryos with three copies of *dpp* (data not shown).

Further evidence that *hnt* and *ush* are early targets of the Dpp signaling pathway was obtained by analyzing transgenic embryos that contain the *dpp*-coding sequence attached to the *eve* stripe 2 enhancer (Fig. 2E). These embryos exhibit both the endogenous *dpp* pattern in the dorsal ectoderm (St Johnston and Gelbart, 1987) as well as an ectopic stripe of expression. The *dpp* stripe results in an expansion in both the *hnt* (Fig. 2D) and *ush* (Fig. 2F) expression patterns. The broadening of these patterns is particularly evident in anterior regions in the vicinity of the *eve* stripe. Increases in *dpp^{+}* gene dose do not expand the *pnr* expression pattern (data not shown). For example, four copies of *dpp^{+}* result in augmented levels of *pnr* expression, but the dorsoventral limits of expression are essentially normal. The stripe2-*dpp* transgene has no obvious effect on the early *sog* and *brk* expression patterns (data not shown).

Brinker represses *pnr* and *tup*

Previous studies have shown that the *pnr* expression pattern expands into neurogenic regions in *brk^{-}* mutant embryos. No

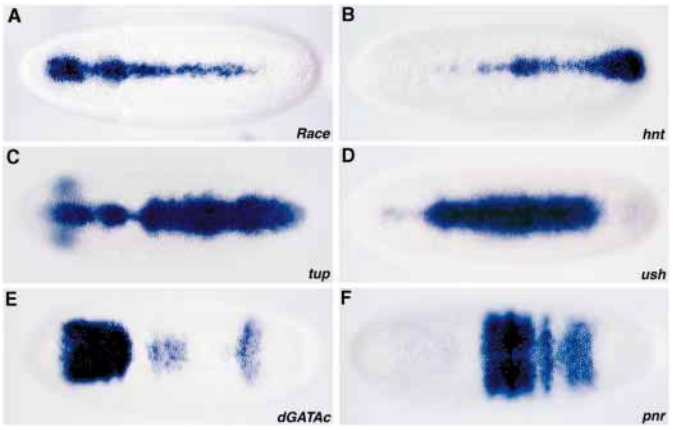


Fig. 1. *dpp* target genes. Dorsal views of wild-type embryos at the initial phase of gastrulation. Embryos were hybridized with digoxigenin-labeled antisense RNA probes and are oriented with anterior to the left. (A) *Race* hybridization probe. Staining is detected in a strip of cells along the dorsal midlne. Staining in anterior regions corresponds to the presumptive brain and optic lobe, while the narrower strip in posterior regions corresponds to the developing amnioserosa. (B) *hnt* hybridization probe. Staining is restricted to a narrow strip at the dorsal midline. The dorsolateral limits of expression are similar to those observed for *Race*, but staining is excluded from anterior regions and extends to the posterior pole. (C) *tup* hybridization probe. Staining is detected in the developing amnioserosa and also extends into dorsal regions of the presumptive dorsal epidermis. (D) *ush* hybridization probe. Staining is detected in a broad band that encompasses the developing amnioserosa and extends into dorsolateral regions of the dorsal ectoderm. This pattern is similar to that observed for *tup* (C) except that the *tup* pattern extends into more anterior regions. (E) *dGatac* hybridization probe. A broad band of staining is detected in anterior regions. There is a stripe near the posterior transverse furrow, and a patch of staining that includes an anterior portion of the amnioserosa. (F) *pnr* hybridization probe. A series of broad stripes are detected throughout the dorsal ectoderm in the posterior half of the embryo.

such expansion was observed for other Dpp/Scw target genes that were previously examined, including *ush* (Jazwinska et al., 1999b). To test the role of the Brk repressor in establishing the different responses of Dpp target genes, the *brk*-coding sequence was attached to the *eve* stripe 2 enhancer.

Transgenic embryos carrying the stripe2-*brk* transgene exhibit both the normal pattern (lateral stripes) in the neurogenic ectoderm (Jazwinska et al., 1999b) as well as an ectopic stripe of expression (Fig. 3B, compare with A). *pnr* is normally expressed in a series of 5 stripes in the dorsal ectoderm (Fig. 3C; Winick et al., 1993). The anteriormost stripe is lost in transgenic embryos carrying the stripe2-*brk* fusion gene (Fig. 3D, compare with C). This result suggests that Brk is sufficient to repress *pnr* in an ectopic location in the embryo.

Additional Dpp/Scw target genes were examined for repression by the stripe2-*brk* transgene. Those showing altered patterns of expression include *tup, rho, hnt* and *Race* (Fig. 3F, data not shown). The normal *tup* expression pattern encompasses both the presumptive amnioserosa and dorsal regions of the dorsal epidermis (Figs 1C, 3E). In transgenic embryos, there is a gap in the pattern in regions where the stripe2-*brk* fusion gene is expressed (Fig. 3F). These results suggest that Brk represses *tup*, even though it appears to respond to a different threshold of Dpp/Scw signaling than *pnr*. Additional experiments were done to determine whether Brk directly represses *tup* expression, or works indirectly by inhibiting Dpp signaling.

Sog inhibition versus Brk repression

To examine the relative contributions of the Sog inhibitor and the Brk repressor in establishing different thresholds of Dpp/Scw signaling activity, target genes were analyzed in *gastrulation defective* (*gd*) mutants that express either a stripe2-*sog* (Ashe and Levine, 1999) or stripe2-*brk* transgene (Fig. 4). Mutant embryos collected from gd^{-}/gd^{-} females lack

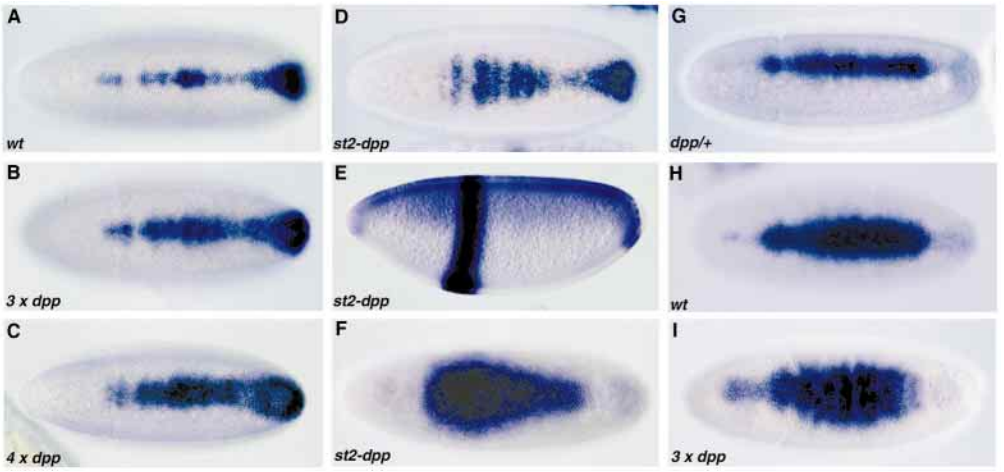


Fig. 2. Altered expression patterns in response to altered dpp^{+} gene dose. Dorsal views (except E) of embryos at the initial phase of gastrulation. A lateral view of the embryo in E undergoing cellularization. The embryos contain different doses of dpp^{+} or express a stripe2-*dpp* transgene. They were hybridized with different digoxigenin-labeled RNA probes and are oriented with anterior to the left. (A-C) *hnt* hybridization probe. Staining is restricted to the dorsal midline in wild-type embryos containing two copies of dpp^{+} (A; see Fig. 1B). The pattern is broader in embryos carrying three copies of dpp^{+} (B) and perhaps even slightly broader in embryos carrying four copies of dpp^{+} (C). (D-F) Transgenic embryos carrying the stripe2-*dpp* fusion gene. *hnt* (D), *dpp* (E) and *ush* (F) expression patterns. The *hnt* and *ush* patterns are substantially broader than those seen in normal embryos, particularly in anterior regions in the vicinity of the stripe2-*dpp* expression pattern. These transgenic embryos contain both the endogenous *dpp* pattern in the dorsal ectoderm and the ectopic stripe 2 pattern (E). (G-I) *ush* hybridization probe. The embryos contain different copies of the dpp^{+} gene. (G) a *dpp*/+ heterozygote; (H) wild-type (2 copies of dpp^{+}); (I) embryo contains three copies of dpp^{+}. There is a progressive expansion of the *ush* expression pattern. The *dpp*/+ heterozygote (G) exhibits an abnormally narrow expression pattern. Genes requiring higher levels of dpp^{+} activity, such as *hnt* and *Race*, are not expressed in these embryos (data not shown).

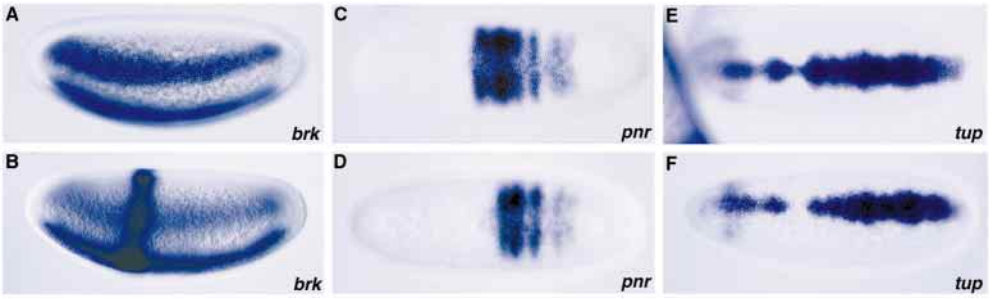


Fig. 3. Brk represses *pnr* and *tup*. Cellularizing embryos were hybridized with *brk*, *pnr* and *tup* RNA probes and are oriented with anterior to the left. (A,B) *brk* expression in a wild-type embryo (A) and transgenic embryo (B) carrying the stripe2-*brk* transgene. In both cases, staining is observed in lateral stripes within the neurogenic ectoderm. In addition, there is an ectopic stripe2 pattern detected in the transgenic embryo (B). (C,D) *pnr* expression in a wild-type (C) and transgenic embryo (D) carrying the stripe2-*brk* transgene. The anteriormost *pnr* stripe is lost in the transgenic embryo (D, compare with C), indicating repression by the stripe2-*brk* fusion gene. (E,F) *tup* expression in a wild-type (E) and transgenic embryo (F) carrying the stripe2-*brk* transgene. The transgene creates a gap in the normal *tup* pattern (F, compare with E).

a Dl nuclear gradient and therefore lack ventral tissues such as the mesoderm and neurogenic ectoderm. All tissues along the dorsoventral axis form derivatives of the dorsal ectoderm, mainly dorsal epidermis (e.g., Roth et al., 1989). Hereafter, we shall refer to such embryos as *gd*$^{-}$. These mutants lack endogenous *sog* and *brk* products, so that the stripe2 transgenes represent the only source of expression. Although the stripe2-*sog* transgene inhibits Dpp signaling, it does not cause activation of *brk* (data not shown).

The *pnr* and *tup* expression patterns are derepressed in *gd*$^{-}$ mutants, and exhibit uniform staining in both dorsal and ventral regions (Fig. 4A,D). These expanded patterns correlate with the expanded expression of *dpp*, which is normally repressed in ventral and lateral regions by the Dl gradient (e.g., Huang et al., 1993). As seen in wild-type embryos (Fig. 3), the stripe2-*brk* transgene represses the anterior portion of the *pnr* expression pattern (Fig. 4B, compare with A). In contrast, the stripe2-*sog* transgene has virtually no effect on the pattern (Fig. 4C). These observations suggest that Brk is the key determinant in establishing the lateral limits of the *pnr* pattern at the boundary between the dorsal ectoderm and neurogenic ectoderm. The failure of stripe2-*sog* to inhibit *pnr* expression might be due to redundancy in the action of the Dpp and Scw ligands. Perhaps either Scw alone or just one copy of *dpp*$^{+}$ is sufficient to activate *pnr*. This would be consistent with the observation that the initial *pnr* expression pattern is essentially normal in *dpp*$^{-}$/*dpp*$^{-}$ and *scw*$^{-}$/*scw*$^{-}$ mutant embryos (data not shown).

The limits of the *tup* expression pattern seem to depend on both Sog and Brk (Fig. 4D-F). The introduction of the stripe2-*brk* transgene leads to a clear gap in the *tup* expression pattern (Fig. 4E), although there is a narrow stripe of repression in *gd*$^{-}$ mutants lacking the transgene (Fig. 4D). The stripe2-*sog* transgene causes a more extensive gap in the *tup* pattern (Fig. 4F). The stripe2-*brk* transgene was also found to repress *Race*, *hnt* and *rho* in this assay (data not shown).

In principle, the gap in the *tup* pattern caused by the stripe2-*brk* transgene (Figs 3F, 4E) could be indirect, and caused by the repression of *dpp*. Previous studies have shown that the early *dpp* expression pattern expands into the ventral ectoderm in *brk*$^{-}$ mutant embryos (Jazwinska et al., 1999b). To investigate this possibility, *tup* expression was monitored in *brk*$^{-}$ embryos, and in wild-type embryos carrying both the stripe2-*brk* and stripe2-*dpp* transgenes (Fig. 5).

The *tup* expression pattern exhibits a transient expansion in *brk*$^{-}$ mutant embryos (Fig. 5C). However, this expansion is only seen in early embryos, prior to the completion of cellularization. By the onset of gastrulation, the pattern is essentially normal (Fig. 5B, compare with A). The stripe2-*brk* transgene creates an early gap in the normal *dpp* expression pattern in wild-type embryos (Fig. 5D). This observation raises the possibility that the repression of *tup* (Figs 3F, 4E) and *rho* (data not shown) is indirectly mediated by the inhibition of Dpp

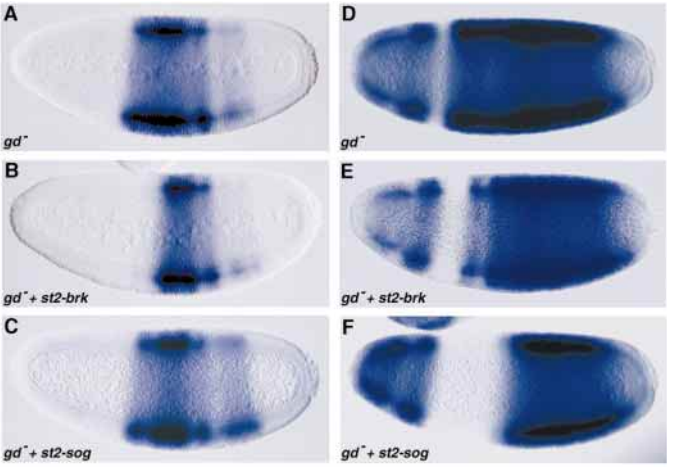


Fig. 4. *pnr* and *tup* expression patterns in *gd*$^{-}$ embryos. Embryos were collected from *gd*$^{-}$/*gd*$^{-}$ females and subsequently hybridized with either the *pnr* probe (A-C) or the *tup* probe (D-F). (A) The *pnr* staining pattern is derepressed in *gd*$^{-}$ embryos. Equally intense staining is observed in dorsal and ventral regions. Staining is excluded from head regions, as seen in wild-type embryos (e.g., Fig. 1F). (B) Same as A, except that the mutant embryo contains the stripe2-*brk* fusion gene. The ectopic Brk stripe represses the anterior portion of the *pnr* pattern (compare with A). (C) Same as A, except that the mutant embryo contains the stripe2-*sog* fusion gene. The ectopic stripe of the Sog inhibitor has little or no effect on the *pnr* pattern (compare with A). (D) The *tup* staining pattern in a *gd*$^{-}$ embryo. Staining is detected in both dorsal and ventral regions. There is small gap in the pattern in the vicinity of the cephalic furrow. (E) Same as D, except that the mutant embryo expresses the stripe2-*brk* transgene. The ectopic stripe of Brk creates a broader gap in the *tup* expression pattern (compare with D). (F) Same as D except that the mutant embryo expresses the stripe2-*sog* transgene. The ectopic Sog stripes creates a broad gap in the *tup* expression pattern.

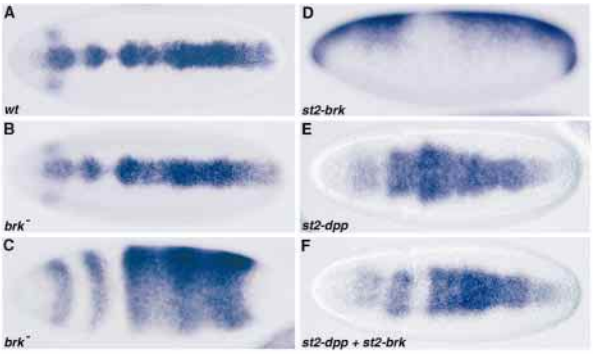


Fig. 5. Brk directly represses *tup*. Embryos were collected from wild-type, mutant and transgenic strains and stained after hybridization with either a *tup* (A-C,E,F) or *dpp* (D) probe. Embryos are oriented as described previously. (A) Dorsal view of a wild-type embryo at the onset of gastrulation. *tup* expression is detected in the presumptive amnioserosa and dorsal regions of the presumptive dorsal epidermis (see Fig. 1C). (B,C) Dorsal views of gastrulating (B) and cellularizing (C) *brk* mutant embryos. The *tup* staining pattern may be slightly broader in the mutant as compared with wild-type embryos at gastrulation (B, compare with A). However, a number of cellularizing embryos exhibit a dramatically expanded *tup* staining pattern (C). This expansion is transient and not detected in older embryos. (D) *dpp* expression pattern in a precellular, transgenic embryo that contains the stripe2-*brk* fusion gene. There is a gap in the *dpp* pattern in regions where the transgene is expressed. (E) *tup* expression pattern in an early gastrulating embryo that contains the stripe2-*dpp* transgene. The *tup* pattern is expanded in anterior regions where the transgene is active (compare with Fig. 2D,F). (F) *tup* expression pattern in an early gastrulating embryo that contains both the stripe2-*dpp* and stripe2-*brk* transgenes. There is a broad gap in the expanded *tup* expression pattern in regions where the transgenes are co-expressed. The gap is more pronounced than the one obtained with the stripe2-*brk* transgene alone (Fig. 3F) since the stripe2-*dpp* transgene directs a broad domain of enhanced Dpp/Scw signaling.

signaling. To test this, the *tup* pattern was examined in embryos carrying both the stripe2-*brk* and stripe2-*dpp* transgenes (Fig. 5F). As expected, the stripe2-*dpp* transgene alone causes a local expansion of the *tup* pattern in the vicinity of the stripe 2 pattern (Fig. 5E). However, the simultaneous expression of both stripe2-*dpp* and stripe2-*brk* leads to a clear gap in the *tup* expression pattern (Fig. 5F). Thus, it would appear that Brk

Fig. 7. Summary of Dpp signaling thresholds. The Dpp/Scw activity gradient presumably leads to a broad nuclear gradient of Mad and Medea across the dorsal ectoderm of early embryos. It is conceivable that the early lateral stripes of *brk* expression lead to the formation of an opposing Brk repressor gradient through the limited diffusion of the protein in the precellular embryo. We have presented evidence that the Dpp/Scw activity gradient might generate as many as four different thresholds of gene activity in the dorsal ectoderm. Peak levels of Dpp and Scw activity lead to the activation of *Race* and *hnt* at the dorsal midline. The *tup* and *ush* patterns represent a third threshold of gene activity. The similar patterns might involve different mechanisms of Dpp signaling since *tup* is repressed by Brk, whereas *ush* is not (data not shown). Finally, the broad *pnr* pattern represents the fourth threshold of gene activity. It is not inhibited by Sog but is repressed by Brk. It is possible that *tup* and *pnr* are differentially repressed by a Brk gradient. Low levels of Brk might be sufficient to direct the lateral limits of the *tup* pattern, whereas high levels may be required to repress *pnr*.

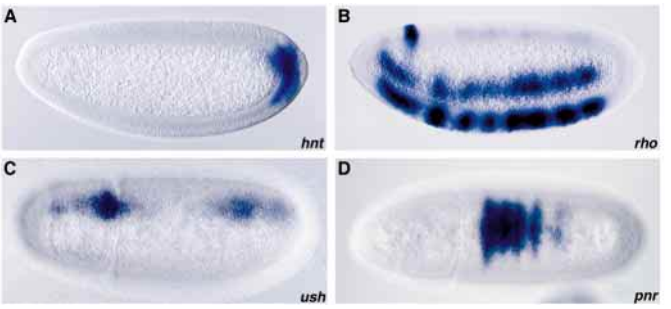
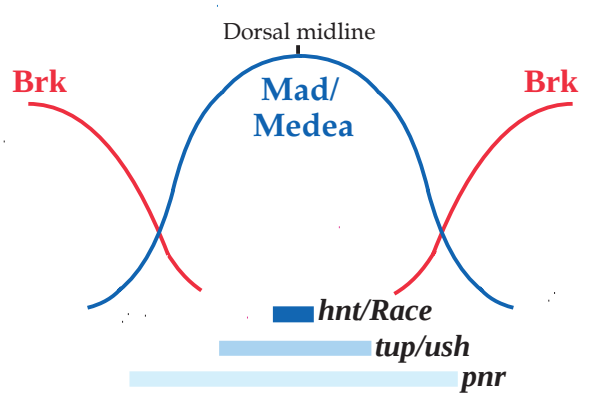


Fig. 6. *hnt*, *rho*, *ush* and *pnr* patterns in *nej*^{1} embryos. Embryos were collected from females that were induced to produce *nej*^{1} germline clones using the *flp*-FRT method. Mutant embryos were hybridized with different digoxigenin-labeled antisense RNA probes. (A) Lateral view of a *nej*^{1} mutant embryo stained with the *hnt* hybridization probe. Expression is restricted to the posterior pole. There is no staining detected in the amnioserosa. (B) Lateral view of a *nej*^{1} mutant embryo stained with the *rho* probe. Normal staining is detected in the lateral, neurogenic regions. However, there is a virtual loss of the dorsal staining pattern. (C) Dorsolateral view of a mutant embryo stained with the *ush* probe. There is a gap in the staining pattern in central regions, and the remaining pattern is narrower than normal. (D) Dorsal view of a mutant embryo stained with the *pnr* probe. There is a narrowing of the pattern as compared with wild-type embryos (see Fig. 1F).

can repress *tup* even in regions containing high levels of Dpp signaling. Similar assays suggest that *Race*, *hnt* and *rho* are not directly repressed by Brk (data not shown).

The CBP coactivator is required for Dpp signaling thresholds

Previous studies have identified mutations in the *Drosophila* homolog of the mammalian CBP histone acetyltransferase gene, *nejire* (Akimaru et al., 1997a). *nej* is maternally expressed so that the detection of early patterning defects depends on the analysis of embryos derived from females containing *nej* germline clones. The complete loss of *nej*$^{+}$ activity results in a failure to make mature eggs. However, it is possible to obtain embryos from a strong hypomorphic allele, *nej*^{1}. These embryos exhibit dorsoventral patterning defects (Akimura et al., 1997b). Recent studies have shown that CBP interacts with Smad proteins (Feng et al., 1998; Janknecht et al., 1998; Topper et al., 1998) including the *Drosophila* protein Mad (Waltzer and Bienz, 1999), a transcription factor downstream of Dpp signaling (reviewed by Raftery and



Sutherland, 1999). In *nej* mutant embryos, there is a loss of the amnioserosa and other derivatives of the dorsal ectoderm (Fig. 6). The expression of target genes requiring peak levels of Dpp signaling is essentially abolished. For example, *hnt* expression is lost in the presumptive amnioserosa (Fig. 6A), but persists at the posterior pole where it might be separately regulated by the torso signaling pathway (see wild-type pattern in Fig. 1B).

There is a similar loss of the dorsal *rho* pattern in mutant embryos (Fig. 6B). In contrast, the lateral, neurogenic stripes are unaffected, indicating that the *nej* mutant does not cause defects in the patterning of the neurogenic ectoderm. Moreover, the fact that the *rho* stripes are excluded from ventral regions, as seen in wild-type embryos, suggests that the patterning of the mesoderm is also normal. Thus, the *nej* mutation does not appear to cause a general loss of transcriptional activation, but instead results in specific patterning defects in the dorsal ectoderm. Target genes that are activated by lower levels of Dpp signaling such as *ush* and *pnr* are also affected by the *nej* mutation (e.g., Fig. 6C,D). In the case of *ush*, there is a loss of staining in central regions of the dorsal ectoderm. Moreover, the residual staining pattern is narrower than the wild-type pattern (Fig. 6C; compare with Fig. 2H). This is reminiscent of the *ush* pattern seen in *dpp*/+ heterozygotes (Fig. 2G). However, the *nej* mutation also causes a narrowing of the *pnr* pattern (Fig. 6D), whereas expression is normal in *dpp*/+ embryos (data not shown).

DISCUSSION

We have presented evidence that the Dpp/Scw activity gradient specifies at least three distinct thresholds of gene activity in the dorsal ectoderm of the early *Drosophila* embryo. These thresholds depend on the interplay between broadly distributed Mad/Medea transcriptional activators and the spatially localized Brk repressor (Fig. 7). Different levels of the Brk repressor help establish distinct limits of *pnr* and *tup* expression. We have also presented evidence that the general CBP coactivator protein plays a surprisingly specific role in the patterning of the dorsal ectoderm, and is particularly important for the regulation of target genes that depend on high and intermediate levels of Dpp/Scw signaling. Despite the fact that CBP has been implicated as a general transcriptional coactivator (e.g., Mannervik et al., 1999), specification of the mesoderm and neurogenic ectoderm appears normal in mutant embryos derived from *nej*^{1} germline clones.

How many thresholds?

The distinct patterns of *hnt*, *tup* and *pnr* expression (Fig. 1) suggest that the Dpp/Scw activity gradient specifies at least three thresholds of gene activity. These genes exhibit differential responses to Dpp dose (Fig. 2) and to the Sog inhibitor (Fig. 4) and Brk repressor (Figs 3-5). It has been previously shown that Brk represses the early patterns of *zen*, *tld* and *dpp* expression (Jazwinska et al., 1999b). However, the initial activation of these genes depends on the maternal Dl gradient and, consequently, they probably do not represent a distinct readout of the Dpp/Scw activity gradient.

The preceding arguments suggest that Dpp and Scw specify three thresholds of gene activity (summarized in Fig. 7). However, only two patterning thresholds, dorsal epidermis and amnioserosa, can be discerned on the basis of analyzing cuticle preparations. It is possible that staggered patterns of *pnr* and *ush* expression define a domain in dorsolateral regions where sensory organs can form. This would be analogous to the specification of the Do sensory bristle in the adult notum. Pnr can activate the pro-neural genes, *achaete* and *scute*, in a broad region of the notum, but expression is restricted to the Do primordium by *ush*, which inhibits Pnr through direct protein-protein interactions (Cubbada et al., 1997; Haenlin et al., 1997).

Transcriptional repression and Dpp signaling thresholds

At least two of the transcription thresholds that are specified by the Dpp/Scw activity gradient depend on the Brk repressor (Fig. 7). In principle, *pnr* can be activated throughout the dorsal half of the embryo but expression is restricted to the presumptive dorsal ectoderm by high levels of the Brk repressor (Jazwinska et al., 1999b; Figs 3, 4 of the present study). In contrast, lower levels of Brk might work together with the Sog inhibitor to specify sharp lateral limits of the *tup* expression pattern (Figs 4, 5). Perhaps the early lateral stripes of *brk* expression permit limited diffusion of the Brk protein in the syncitial nuclei of precellular embryos. Such diffusion might produce a Brk repressor gradient that is complementary to the Dpp/Scw signaling gradient. Presumably, Sog-Dpp/Scw interactions create a gradient of activated Mad/Medea complexes in the nuclei of the dorsal ectoderm (reviewed by Raftery and Sutherland, 1999; Fig. 7). Perhaps the lowest levels of Brk that can repress *tup* correspond to the lowest levels of the Mad/Medea gradient that can activate it. According to this view, the Brk gradient functions redundantly with the Mad/Medea gradient to specify the lateral limits of the *tup* expression pattern. This would be similar to the specification of the posterior border of *eve* stripe 2. This border depends on both limiting amounts of the anteroposterior gradient of Bicoid (Bcd) activator and on the Krüppel (Kr) repressor gradient emanating from central regions of the embryo. Stripe2-*lacZ* fusion genes lacking critical Kr repressor sites exhibit only a slight posterior expansion of the stripe border due to limiting amounts of Bcd. The interplay between Bcd and Kr helps produce a sharp stripe border (Stanojevic et al., 1991; Small et al., 1992). Similarly, it is conceivable that Mad/Medea and Brk produce sharp borders of *tup* expression (see Fig. 3E).

Thus, the situation we observe in the dorsal ectoderm of early embryos is quite similar to the patterning of wing imaginal disks. A Dpp activity gradient at the A/P compartment boundary of the wing disk establishes different limits of *spalt* (*sal*) and *optomotor blind (omb)* expression. High levels of the Brk repressor specify the lateral limits of the broad *omb* pattern, whereas the combination of low levels of Brk and intermediate levels of Dpp signaling regulate the narrower *sal* pattern (Campbell and Tomlinson, 1999; Jazwinska et al., 1999a). A difference between the wing disk and embryo is that there are target genes in the embryo which are activated by peak levels of Dpp signaling (e.g., Race and hnt) but not repressed by Brk. Such genes have not yet been described in the wing disk.

The role of CBP in establishing Dpp/Screw signaling thresholds

A remarkable number of genes are required for producing and

receiving the Dpp/Scw gradient in the dorsal ectoderm (reviewed by Podos and Ferguson, 1999). It would appear that an increasing number of nuclear factors are also required for generating different thresholds of gene activity in response to this gradient. In addition to Mad/Medea and Brinker, we have obtained evidence that the general transcriptional coactivator, CBP, is essential in this process. Previous studies have shown that the expression of a *Race-lacZ* fusion gene is lost in the presumptive amnioserosa of mutant embryos derived from nej^1 germline clones (Waltzer and Bienz, 1999). Here we have shown that there is an extensive, but specific, loss of dorsal ectoderm derivatives in these mutants. There is a loss of high Dpp/Scw signaling thresholds including *Race*, *hnt* and *rho*. Moreover, target genes that are activated by intermediate levels of Dpp/Scw signaling, such as *ush*, exhibit abnormally narrow patterns of expression. However, unlike *dpp*/+ heterozygotes or scw^{-}/scw^{-} homozygotes there is also a narrowing of the *pnr* expression pattern in *nej* mutants.

These disruptions in Dpp patterning thresholds may reflect relatively specific CBP-Mad interactions in the early embryo. It is possible that Mad recruits CBP to the promoter regions of Dpp target genes (Waltzer and Bienz, 1999). Alternatively, CBP-mediated chromatin decondensation may be essential for the binding of Mad/Medea to chromatin templates in vivo. The promoter regions of *hnt* and *rho* might contain low-affinity Mad/Medea operator sites and thereby mediate activation only by high levels of Dpp signaling. Such genes would be expected to exhibit particular sensitivity to reductions in CBP activity. The *pnr* promoter region might contain optimal, high-affinity Mad/Medea operator sites. The narrowing of the *pnr* pattern in *nej* mutants might indicate that CBP is required for the efficient occupancy of even optimal sites in dorsolateral regions where there are diminishing levels of Dpp/Scw signaling.

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