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EGF receptor signaling induces *pointed P1* transcription and inactivates Yan protein in the *Drosophila* embryonic ventral ectoderm

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SUMMARY

The induction of different cell fates along the dorsoventral axis of the *Drosophila* embryo requires a graded activity of the EGF receptor tyrosine kinase (DER). Here we have identified primary and secondary target genes of DER, which mediate the determination of discrete ventral cell fates. High levels of DER activation in the ventralmost cells trigger expression of the transcription factors encoded by *ventral nervous system defective* (*vnd*) and *pointed P1* (*pntP1*). Concomitant with the induction of *pntP1*, high levels of DER activity lead to inactivation of the Yan protein, a transcriptional repressor of Pointed-target genes. These two antagonizing transcription factors subsequently control the expression of secondary target genes such as *otd*, *argos* and *tartan*. The simultaneous effects of the DER pathway on *pntP1* induction and Yan inactivation may contribute to the definition of the border of the ventralmost cell fates.

Key words: *Drosophila*, EGF receptor, Pointed, Yan, *spitz* group, Cell fate, *ventral nervous system defective*

INTRODUCTION

The *Drosophila* EGF receptor (DER), a receptor tyrosine kinase, is required in multiple sites during development. In some contexts DER was shown to mediate binary decisions, e.g. the generation of posterior follicle cell fates (González-Reyes et al., 1995; Roth et al., 1995). In other cases however, different levels of DER activity are correlated with the induction of multiple cell fates in a given tissue. High levels of DER activity are required for the formation of wing veins, while intermediate levels are responsible for determining cell morphology and size in the intervein regions (Diaz-Benjumea and Hafen, 1994). During embryonic development the DER cascade represents the zygotic pathway responsible for patterning the embryonic ventral ectoderm. High levels of DER activity induce the ventralmost cell fates, while lower levels are required to induce ventrolateral cell fates (Schweitzer et al., 1995b).

In view of the ability of the DER pathway to induce several cell fates within a given tissue, it is important to elucidate the mechanisms responsible for the formation and interpretation of graded DER activity. Analysis of DER signaling has been aided by the fact that the activity of RTKs is mediated via the common, well conserved Ras signaling cascade. Within the cell, activation of the Sos/Ras/MAP kinase pathway by DER has been demonstrated (Diaz-Benjumea and Hafen, 1994; Schweitzer et al., 1995b).

Further understanding of DER regulation was facilitated by the identification of a class of mutants (termed the *spitz* group) that display similar phenotypes to *DER* and genetically interact with it (Mayer and Nüsslein-Volhard, 1988; Sturtevant et al., 1993; Raz and Shilo, 1993). Spitz is a DER ligand, which is produced as a transmembrane precursor (Rutledge et al., 1992). Its processing to generate the active, secreted form is the limiting event in several instances of DER function (Schweitzer et al., 1995b). Rhomboid and Star, two novel transmembrane proteins (Bier et al., 1990; Kolodkin et al., 1994), modulate the level of DER activity, possibly by facilitating Spitz processing (Schweitzer et al., 1995b).

Finally, based on the analysis of the mutant cuticle phenotype, a requirement for *pointed* (*pnt*) in patterning the embryonic ventral ectoderm, as a member of the *spitz* group, has been suggested (Mayer and Nüsslein-Volhard, 1988). The *pnt* gene harbors two promoters separated by 50 kb, which generate two alternative transcripts encoding transcription factors of the ETS family: Pointed P1 acts as a constitutive transcriptional activator, while Pointed P2 requires phosphorylation by MAP kinase to become a potent transcriptional activator (Klämbt, 1993; Klaes et al., 1994; O'Neill et al., 1994; Brunner et al., 1994). *pntP1* is normally expressed in the ventral ectoderm and tracheal cells. *pntP2* is expressed in the mesoderm and midline glial cells, and was shown to be a crucial downstream target for DER in the midline glial cells (Scholz, H., Sadlowski, E., Klaes, A. and Klämbt, C., unpublished data). An inhibitory ETS type transcription factor, which competes with Pointed for binding of common sites on the DNA, has been identified and termed Yan (Lai and Rubin, 1992; O'Neill et al., 1994; Brunner et al., 1994; Tei et al., 1992). Phosphorylation of Yan by MAP kinase induces its translocation from the nucleus to the cytoplasm (Rebay and Rubin, 1995).

A novel feature of the DER signaling cascade is an

inhibitory secreted polypeptide, Argos, which is likely to serve as an antagonistic ligand of DER (Freeman et al., 1992; Schweitzer et al., 1995a). *argos* transcription is induced by the DER pathway (Golembo et al., 1996a). It thus serves as an inhibitory feedback loop: the cells exhibiting the highest level of DER activation express Argos, which is secreted and could serve to terminate or reduce DER signaling in the neighboring cells. Argos therefore appears to be required for defining a restricted time window of DER signaling and for preserving the graded effects of DER activation.

This work examines the transcriptional responses to DER activation during development of the embryonic ventral ectoderm. Two cohorts of DER-target genes were identified. Primary targets (*pntP1*, *vnd* or *fas*III) are induced in different ectodermal domains. Secondary target genes of DER (*otd*, *argos* and *tartan*) are activated by Pointed P1 in response to DER signaling. The proper induction of these genes requires the concomitant inactivation of Yan, mediated by DER signaling.

MATERIALS AND METHODS

DNA probes and antibodies

To analyze *pointed* expression a general *pnt* probe as well as *pntP1*- and *pntP2*-specific cDNA fragments were used (Klämbt, 1993). *otd* cDNA (Finkelstein et al., 1990), *argos* cDNA (Freeman et al., 1992), *tartan* cDNA (Chang et al., 1993) and *yan* cDNA clones (Lai and Rubin, 1992) were used to monitor the respective gene expression. Monoclonal anti-FasIII antibodies (obtained from T. Volk) were used and polyclonal anti-βGal rabbit antibodies (Cappel) were utilized to detect the balancer chromosomes.

Staining

In situ hybridization experiments have been carried out with digoxigenin-labeled cDNA probes as described (Tautz and Pfeifle, 1989), with minor modifications. A detailed protocol is available on request. Antibody stainings have been performed according to Klämbt et al. (1991).

Fly lines

The following mutant lines have been used: pnt^{8B74} (Jürgens et al., 1984), $pnt^{\Delta 88}$ and pnt^{rM254} (Klämbt, 1993; Scholz et al., 1993). The mutation $pnt^{16.1}$ is an additional EMS-induced allele (Scholz, unpublished data) which like pnt^{8B74} and $pnt^{\Delta 88}$ represents an amorphic *pointed* mutation. Amorphic *yan* alleles aop^{II5} and aop^{I5} (Nüsslein-Volhard et al., 1984; Rogge et al., 1995) gave essentially the same results in our experiments. The allele yan^{e2d} (Rogge et al., 1995) behaved as a semi cold-sensitive, and showed an amorphic *yan* phenotype only at 18°C. The flb^{1F26} allele was used at 29°C in which it is amorphic (Raz and Shilo, 1992). The double mutant yan^{e2d}, flb^{1F26} chromosome was generated by recombination and the embryos maintained at 29°C. A fly strain directing Gal4 expression in the *Krüppel* domain (*Kr*-Gal4) was provided by M. Leptin, the *rho*-Gal4 strain was provided by M. Levine, UAS-*pntP*1.0 was isolated in a local jump experiment originating from the insertion UAS-P1.3 on the third chromosome (Klaes et al., 1994), a UAS-activated Yan line was provided by I. Rebay (Rebay and Rubin, 1995) and a UAS-s*Spi*4a integrated on the second chromosome was used (Schweitzer et al., 1995b).

RESULTS

Pattern formation in the ventral ectoderm

Pattern formation in the ventral ectoderm has been reported to depend on the action of the *spitz* group genes and the *Drosophila* EGF receptor homologue (DER). In *spitz* group mutants as well as in *flb*/*DER* embryos, loss of ventral cell fates is observed (Mayer and Nüsslein-Volhard, 1988; Raz and Shilo, 1993). By mechanisms discussed below, the activity of these proteins is converted into several discrete cell fates, which can be identified at stage 10/11 by the expression of various markers. The ventralmost 1-2 cell rows on each side of the midline express *orthodenticle* (*otd*) (Wieschaus et al., 1992), *argos* (*aos*) (Freeman et al., 1992), *tartan* (*trn*) (Chang et al., 1993), *ventral nervous system defective* (*vnd*) (Jimenez et al., 1995) and *fas*III (Patel et al., 1987). The ventrolateral 2-3 cell rows express only *fas*III (schematized in Figs 1, 3A-C).

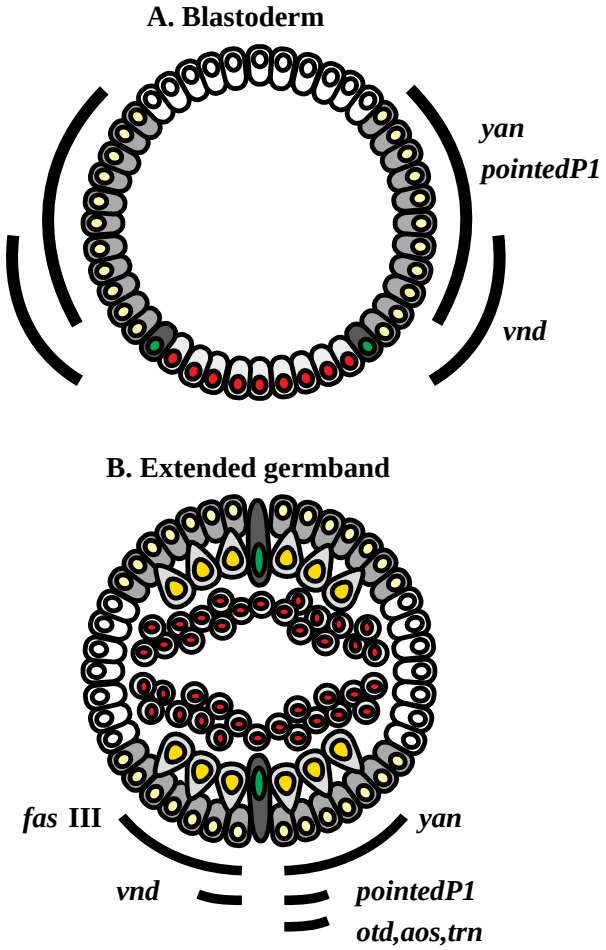

Fig. 1. A scheme for expression of markers in the ventral ectoderm. Schematic summary depicting the expression domains of the following genes: *yan*, *pointed P1*, *ventral nervous system defective* (*vnd*), *fasciclinIII* (*fasIII*), *orthodenticle* (*otd*), *argos* (*aos*) and *tartan* (*trn*). Only expression in the neuroectoderm is shown. Gray shading and yellow nuclei indicate the neurogenic region or neuroectoderm. Nuclei of delaminating neuroblasts are in bright yellow. Dark gray shading and green nuclei indicate the mesectoderm. The mesoderm is indicated by red nuclei. (A) During the cellular blastoderm (stage 5/6), *yan* and *pointed* are expressed in the neuroectoderm. Expression at the ventral margin of the neuroectoderm is lowest. *vnd* is expressed in the ventral region of the neuroectoderm. (B) At the extended germband stage (stage 10), the neuroblasts segregate. *yan* expression is now found in a broad ventral region, whereas *pointed*, *otd*, *argos*, *trn* and *vnd* are expressed in a two-cell-wide row of cells. FasIII is expressed by 4-5 cell rows (in the medial and mediolateral cells). The hierarchy of the different genes is discussed in the text.

Generating a perpendicular gradient of DER activation

Ectopic expression of secreted Spitz resulted in expression of *otd* within the entire ventral ectoderm, suggesting that ventral expression of *otd* is normally induced by higher levels of DER activity (Schweitzer et al., 1995b). *otd* expression does not extend to the dorsal ectoderm under these conditions, presumably because dorsal fates have already been specified by the Dpp pathway. Since it was not possible to directly visualize the putative graded distribution of DER activity, we have generated a gradient of DER activation perpendicular to the dorsoventral axis, by expressing secreted Spitz in the *Krüppel* (*Kr*) domain (see experimental procedures and Fig. 2A). *Kr* is expressed in parasegments T2-A4. In the T1/T2 boundary, where *Kr* expression terminates, an anterior-posterior gradient of DER activation should be generated by graded reduction in *Kr* levels and the diffusion of secreted Spitz. Induction of *lacZ* expression in the *Kr* domain was followed. At stage 9 a patchy expression was detected in the ventral ectoderm. However, *Kr* expression in the T1/T2 boundary terminates in the same row of cells along the anterior-posterior axis (Fig. 2B).

The cells in the intersection between the two gradients are likely to monitor the cumulative effects of secreted Spitz. Their response should thus reveal the nature of the normal gradient of DER activation in the dorsoventral axis. On each side of the midline, expression of *otd* in this region appears to form a 'wedge-like' shape, with ~8-10 cells on each axis (Fig. 2C). This symmetrical response of the cells to the endogenous DER activity, and the artificially induced gradient of DER activation, suggests that, in wild-type embryos, a dorsoventral gradient of DER activation is operating. This gradient is likely to reflect the availability of secreted Spitz, which may be generated in the ectoderm in a restricted pattern or alternatively could emanate from the ventral midline (Golembo et al., 1996b). This paper will examine how the graded DER signal is converted into distinct domains of ventral cell fates.

Pointed is required for ventral ectoderm formation

Previous work has shown that ventral cell fates, as monitored by the expression of *otd* and *argos*, depend on the DER pathway (Schweitzer et al., 1995b; Golembo et al., 1996a). Ventral pattern formation also depends on the *spitz* group gene *pointed* (Mayer and Nüsslein-Volhard, 1988). Of the two *pointed* transcripts, only *pntP1* is expressed in the ventral ectoderm. It first appears prior to gastrulation in the entire neuroectoderm region. As gastrulation commences, *pntP1* expression is confined to the 2-3 ventralmost cell rows. By stage 10, the transcript is restricted to 1-2 cell rows in the ventral ectoderm, and disappears by stage 11/12 (Klämbt, 1993; Fig. 4A).

To further analyze the function of Pointed in ventral patterning, we assayed the expression of several markers in *pnt* mutant embryos. The genes *otd*, *argos* and *trn* are expressed at stage 10/11 in the ventralmost 1-2 cell rows (see Figs 1, 3A-C). In *pnt* null mutant embryos, the expression of *otd*, *argos* and *trn* in these cells is abolished or significantly reduced (Fig. 3D-F).

To determine whether Pointed is sufficient for the induction of these genes, ectopic expression of *pntP1* was induced by *Kr*-Gal4. For all three markers, induction of expression in the *Kr* domain was observed (Fig. 3G-I), suggesting a direct transcriptional activation of these three genes by Pointed P1. Other markers normally expressed in a similar pattern in the ventralmost cells (e.g. *vnd*) are not affected by ectopic Pointed P1 expression. This strengthens the notion that the expanded expression of *otd*, *argos* and *trn* does not represent a secondary response to a general cell fate alteration induced by Pointed. Expression of Pointed P2, the non-constitutive form of

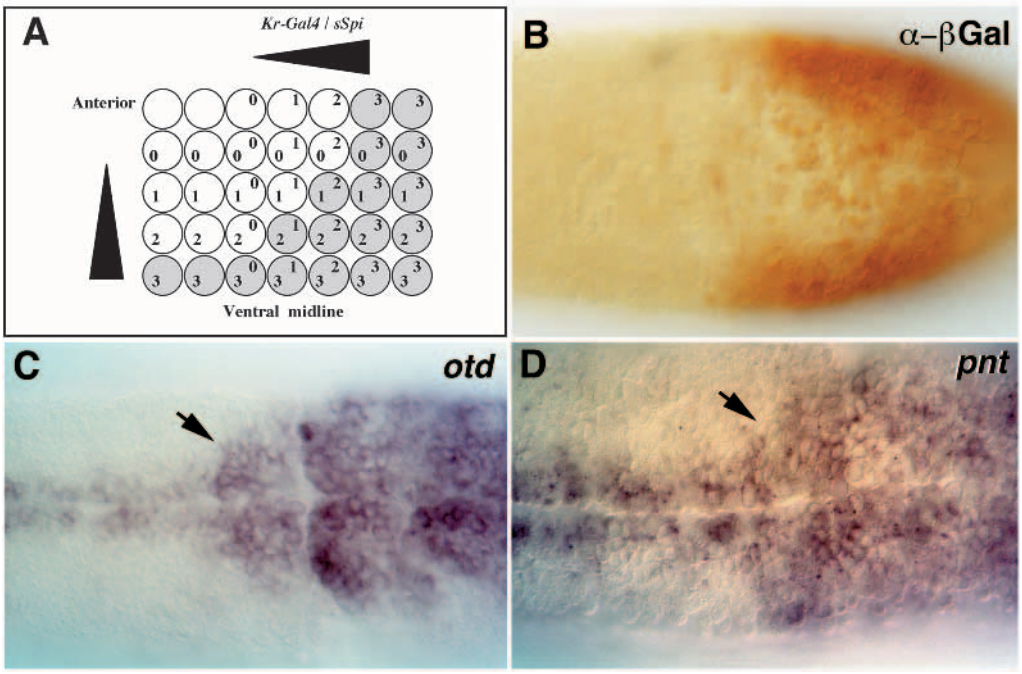

Fig. 2. Perpendicular gradients of DER activation generate 'wedge'-like responses. (A) A scheme for the effects of the endogenous gradient of secreted Spitz and the perpendicular gradient induced by *Kr*-Gal4/UAS-*secreted spitz*. Expression of *otd* reflecting the ventralmost cell fate is monitored. Arbitrary units of DER activation, contributed by each of the two gradients, are shown. When the sum equals, or is greater than 3, expression of *otd* is induced (shaded circles). The resulting pattern is a 'wedge' of *otd* expression in the intersection between the gradients, on each side of the midline. (B) Expression of *Kr*-Gal4 as monitored by induction of UAS-*lacZ* in a stage 9 embryo, viewed from the ventral side. (C) Expression of *otd* in a *Kr*-Gal4/UAS-*sspi* embryo at stage 11 (ventral view). Note the formation of the 'wedge' shape, representing the domain of highest DER activity (arrow). (D) Expression of *pnt* in a *Kr*-Gal4/UAS-*sspi* embryo stage 9 (ventral view, arrow marks the wedge). Note: in all figures anterior is to the left.

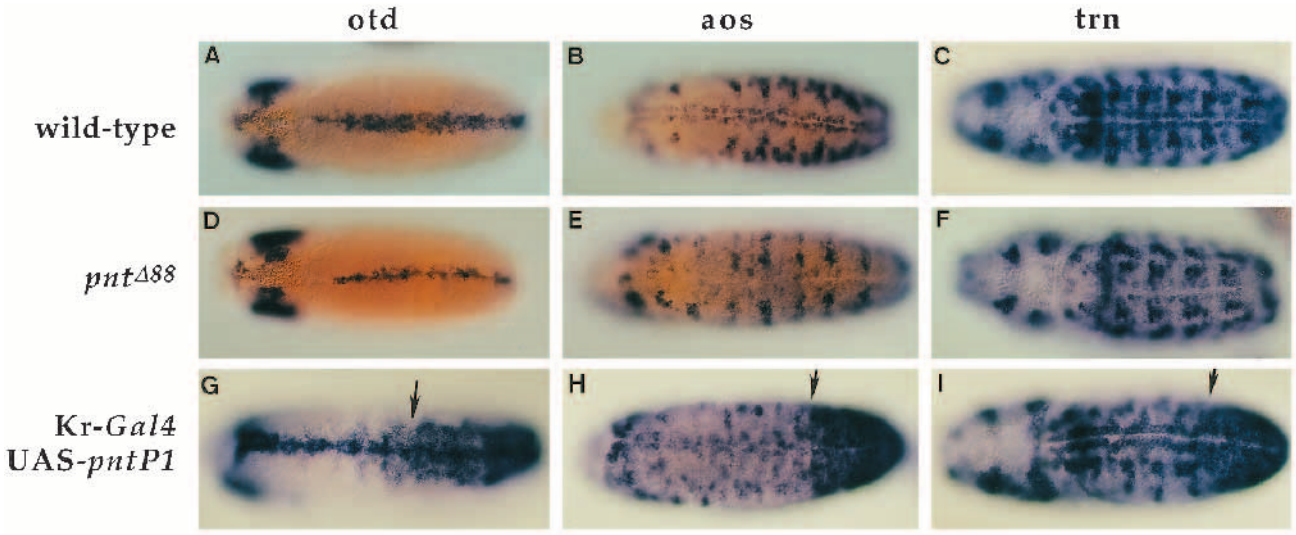

Fig. 3. Pointed P1 is required for induction of the ventralmost cell fates. Expression of *otd*, *argos* and *trn* in wild-type embryos (A-C), mutant *pointed* embryos (D-F) and embryos expressing *pntP1* ectopically in the *Krüppel* expression domain (G-I). (A) In a stage 11 embryo, *otd* is expressed in two cell rows flanking the midline cells, as well as in two large patches of cells in the head. (B). In a stage 11/12 embryo, *argos* expression is found in one to two cells rows flanking the midline as well as in segmental stripes along the dorsoventral axis. (C) *trn* transcripts in a stage 11 embryo are found in segmental stripes in the dorsoventral axis as well as in a 1-2 cell wide stripe of cells flanking the ventral midline. (D) In mutant $pnt^{\Delta 88}$ embryos, transcription of *otd* appears significantly down-regulated in the ventral ectoderm, whereas expression in the head region as well as in the midline cells appears unaffected. In double mutant embryos homozygous for both $pnt^{\Delta 88}$ and yan^{aopII5}, a similarly reduced expression of *otd* is observed. Thus, the residual expression of *otd* observed in $pnt^{\Delta 88}$ embryos does not appear to be regulated by another ETS domain transcriptional activator. (E) Expression of *aos* is more drastically affected in $pnt^{\Delta 88}$ embryos. In stage 11 embryos, no *aos* expression can be detected in the cell rows flanking the ventral midline. Although the stripe-like *aos* expression along the dorsoventral axis is still detectable in homozygous $pnt^{\Delta 88}$ embryos, some lateral expression of *aos* also appears to be under the control of *pointed*. (F) Expression of *trn* in $pnt^{\Delta 88}$ embryos is characterized by a lack of expression in the ventralmost ectodermal cells. (G-I) To investigate whether *pntP1* expression is sufficient to direct the expression of *otd*, *aos* and *trn*, we analyzed their expression in *Kr*-Gal4/UAS-*pntP1* embryos. Expression of (G) *otd*, (H) *aos* and (I) *trn* following ectopic expression of *pntP1*. In all cases, expression correlates with the expression of *Kr* (arrows show the border of the *Kr* domain). (A,C,D,F,I) Dorsal views; (B,E,G,H) ventral views.

Pointed, in the *Kr* domain did not give rise to an ectopic induction of target genes such as *otd* (not shown).

DER signaling induces *pointedP1* transcription

In view of the above results, we wanted to ask whether the previously observed effects of DER on *otd* and *argos* expression are mediated by Pointed. Since Pointed P1 is thought to be a constitutively active transcription factor, with no requirement for modulation of its activity by the DER/MAP kinase signaling pathway, a direct induction of *pntP1* transcription appeared possible. The early onset of *pntP1* expression takes place prior to the time in which the DER pathway is required to pattern the ectoderm (Raz and Shilo, 1993). Since early *pntP1* expression in the ectoderm is maintained in *flb*/*DER* mutant embryos (not shown), it is clearly not DER dependent. However, at stage 9/10, prior to the appearance of *pntP1* in the tracheal pits, expression of *pnt* is not observed in the ventral ectoderm of *flb*/*DER* mutant embryos (Fig. 4B), indicating the transition of *pntP1* expression to a DER-dependent mode.

In a complementary experiment, activation of DER by expression of secreted Spitz in the *Kr* domain, resulted in the induction of *pnt* expression in the same region (Fig. 4C). A 'wedge' of *pnt* expression was observed in the T1/T2 boundary, similar to the one observed for *otd* (Fig. 2D). Utilization of *pointed*-specific probes demonstrated that only the *pntP1* transcript is induced by secreted Spitz (Fig. 4D).

Yan is required for patterning the ventral ectoderm

In view of the essential role of Pointed in patterning the ventral ectoderm, it was important to determine the function of *yan* which encodes a negative regulator of ETS transcriptional activators. *yan* expression in the embryo is first detected at stage 5/6 where it is found in the dorsal ectoderm (Fig. 5A). *yan* expression declines in a dorsal-ventral gradient and is not found in the mesoderm (Fig. 5B). To define the ventral borders of *yan* expression, we performed in situ hybridization experiments using *yan* and *single minded* (*sim*) probes. *sim* expression demarcates the developing midline cells directly flanking the mesoderm; *yan* expression is not observed in the presumptive midline or in the mesodermal cells up to stage 10. At stage 8, *yan* expression is found in the head and marks a broad region in the thoracic and abdominal segments, again excluding the mesoderm and midline cells. This broad ectodermal expression is maintained until stage 10/11 (Fig. 5C), when it is reduced and appears more pronounced in the tracheal pits. From stage 11 onwards, *yan* transcripts are also detected in the midline cells (Scholz, H., Sadlowski, E., Klaes, A. and Klämbt, C., unpublished data). Expression of *yan* does not depend upon DER, as it is unaltered in *flb*/*DER* embryos, or in

embryos in which the expression of secreted Spitz is induced by *Kr*-Gal4 (not shown).

The function of *yan* in patterning the ectoderm was investigated by examining the expression of *otd*, *argos* and *trn* in *yan* mutant embryos. A clear expansion of the ventralmost markers *otd*, *argos* and *trn* is observed, such that 5-8 cell rows on each side of the midline are expressing these genes (Fig. 5D-F). This expanded region is much broader than the zone in which the DER-dependent induction of *pntP1* transcription takes place at stage 9/10. In addition, all three analyzed genes showed increased levels of expression in *yan* mutant embryos. Absence of the Yan protein may thus allow the Pointed P1 protein, which is expressed earlier in a broader domain, to efficiently induce ventralization. This suggestion was confirmed by analysis of embryos that were mutant for both *yan* and *flb*. In these embryos, expression of *otd* in a pattern that is broader than the wild-type one is observed (not shown). Therefore, in the absence of DER signaling and Yan, the early DER-independent expression of *pntP1*, is capable of triggering *otd* expression.

In a complementary experiment, the activated form of Yan, which is unable to undergo phosphorylation by MAP kinase (Rebay and Rubin, 1995), was expressed in wild-type embryos. Indeed, the expression of *otd* and *argos* was significantly reduced or abolished, in the region where activated Yan was expressed (Fig. 5G-I).

pnt transcription does not appear to be regulated by Yan, since no alteration in the expression of *pntP1* was observed in *yan* mutant embryos (not shown). Furthermore, *pnt* expression appeared unchanged in two EMS-induced amorphic *pnt* alleles (pnt^{8B74} and $pnt^{16.1}$), suggesting that no autoregulatory feedback loop is controlling *pnt* expression.

Additional candidate primary genes triggered by DER

The response of the ventralmost cells to DER activation described above, occurs in two phases. First, DER triggers the expression of *pntP1*. Subsequently, Pointed P1 in conjunction with reduced Yan levels, triggers the expression of the second wave of genes. The initial response to DER activation may include other genes in addition to *pnt*. One candidate is *vnd*, which was shown to be expressed in the ventralmost cells (Jimenez et al., 1995; Mellerick and Nirenberg, 1995). The level of *vnd* expression is not reduced in *pnt* mutant embryos (not shown). To test if *vnd* is triggered by DER, expression of the gene was monitored in *flb*/*DER* mutant embryos. Indeed, while the early phases of expression are not affected, no expression of *vnd* is detected from stage 11 in the mutants (not shown). Following induction of secreted Spitz by *Kr*-Gal4, ectopic expression of *vnd* in the ventral ectoderm is observed (Fig. 4F).

Another class of genes that are likely to be triggered directly by the DER pathway are the ones expressed in a broader domain of the ventral ectoderm, which includes ventrolateral cells in which the DER-dependent expression of *pntP1* does not take place. *fas*III is a candidate for this category of genes. Expression of FasIII in 4-5 cell rows of the ventral ectoderm on each side of the midline requires the activity of DER (Raz and Shilo, 1993) (see Fig. 1). The FasIII domain is also expanded following hyperactivation of DER (Schweitzer et al., 1995b). The expression of FasIII was examined in different backgrounds of *pnt* and *yan*. No alterations in the pattern of *fas*III expression were detected in pnt^{8B74} or yan^{e2d} mutant embryos and embryos in which the expression of *pntP1* was induced by *Kr*-Gal4 (not shown). The results indicate that expression of *fas*III, a representative DER-dependent ventrolateral marker, does not appear to be regulated by Pointed or Yan. Previous experiments have also documented in *pnt* mutant embryos only weak effects on the expression of other ventrolateral markers (Kim and Crews, 1993).

DISCUSSION

DER activation gradient

In the developing embryonic ventral ectoderm, the DER RTK is expressed in a uniform pattern (Zak et al., 1990). By the generation of a perpendicular gradient of DER activation, we have shown that DER is triggered in a graded manner along the dorsoventral axis. The source of this gradient may lie in graded Spitz processing in the ectoderm. Alternatively, specific signals may emanate from the midline and generate a graded response of DER (Kim and Crews, 1993; Golembo et al., 1996b). The DER activation gradient is inherently different from other gradients that were previously described. In the case of the Bicoid or Dorsal gradients, for example, cooperative responses

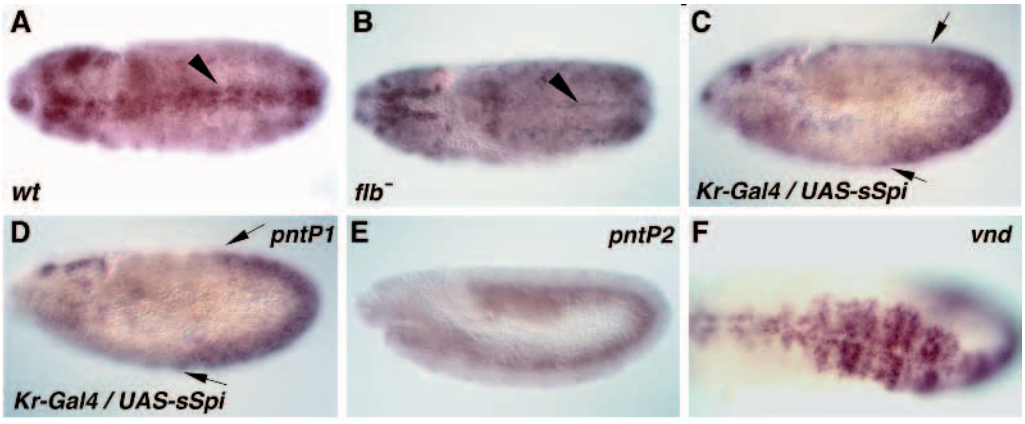

Fig. 4. *pointed P1* transcription is induced by DER. (A). In a wild-type embryo (stage 10), expression of *pnt* in the ventralmost 1-2 cell rows (arrowhead). (B) In a homozygous flb^{1F26} embryo (stage 10), *pnt* staining in the ventral cells is diminished (arrowhead). (C) In a *Kr*-Gal4/UAS-*sspi* embryo (stage 9), expanded expression of *pnt* is observed in the *Kr* domain (marked by arrows). (D) Staining of a similar embryo with a *pntP1*-specific probe shows the same result. (E) Staining with a *pntP2*-specific probe shows only the normal expression in the mesoderm, without ectopic expression in the *Kr* domain. (F) Staining of a similar embryo (stage 10/11) with a *vnd* probe shows expanded expression within the *Kr* domain, in the ventral region. A and F are ventral views, B is dorsal and C-E are lateral views.

to their nuclear concentration account for the generation of borders (St. Johnston and Nüsslein-Volhard, 1992; Jiang and Levine, 1993). These responses are stable over time, as long as the initial concentration of the morphogen is maintained.

In contrast, DER possesses a catalytic tyrosine kinase domain and activates a cytoplasmic cascade of kinases. Thus, graded DER activation generates a gradient of enzymatic activities. The resulting responses to these activities are not fixed over time. Cells in which a lower level of DER activation takes place, may eventually reach the same 'state' as the cells in which the highest level of DER activation has commenced. This temporal constraint highlights the requirement for Argos as a negative element, which is induced by the DER pathway to terminate signaling and maintain the graded response to DER activation (Golembo et al., 1996a). The downstream consequences of DER activation, which display distinct borders of gene expression, should therefore reflect the response to the enzymatic activity of the DER pathway.

DER induces *pointedP1* expression

This work has identified *pntP1* as a central response element of high DER activity in the developing ectoderm. Although *pntP1* has an early embryonic DER-independent expression phase, it becomes transcriptionally dependent upon DER at stage 9/10. Subsequently, Pointed P1 mediates (directly or indirectly) the induction of several transcriptional responses to high DER activity. Induction of *argos* by Pointed P1 in the ventralmost cells followed by Argos diffusion, results in a shutdown of DER signaling. Thus, the simultaneous transcriptional induction of positive and negative elements, generates a time window of DER activity, which can last until the Argos protein is produced and secreted at effective concentrations.

DER inactivates Yan

The cellular consequences of DER signaling in the ectoderm are further modulated by the post-translational modification of the Yan protein. Yan, which like Pointed is an ETS domain protein, was shown to counteract the positive effects of Pointed, presumably by competing for the Pointed-binding site(s) on target genes (O'Neill et al., 1994). In regions of high DER activity, Yan is likely to be inactivated by MAP kinase phosphorylation. In *C. elegans*, *lin*-1 encodes an inhibitory ETS-domain protein (Beitel et al., 1996) and was placed in the Let-23 EGF receptor pathway downstream to MAP kinase (*sur*-1) (Wu and Han, 1994).

The ventralization of *yan* mutant embryos is dramatic. Up to eight cell rows express the ventralmost markers on each side of the midline. This ventralization is wider than the domain in which *pointed* is induced by DER, and may reflect the response to the initial, DER-independent and broader domain of *pntP1* expression. Thus, Yan may normally serve to restrict the early activity of Pointed. Only upon triggering of the DER pathway is *pntP1* transcription induced in the ventralmost cells, and its activity manifested in the region where Yan is efficiently inactivated.

Yan appears to have an antagonistic interaction with the DER pathway in other instances as well. Expression of Yan in the embryonic dorsal head ectoderm blocks cell divisions, and allows the neuronal and ectodermal differentiation of these cells to take place. In the eye imaginal disc, Yan blocks cell proliferation, which is also likely to be driven by DER, and permits neuronal differentiation to commence (Rogge et al., 1995). Inactivation of transcriptional repressors by an RTK, subsequently leading to transcriptional induction, was also demonstrated recently for Torso, which triggers phosphorylation of NTF-1/Grainyhead (Liaw et al., 1995).

Conversion of the DER gradient to distinct domains – further refinement

Several features responsible for the restricted induction of ven-

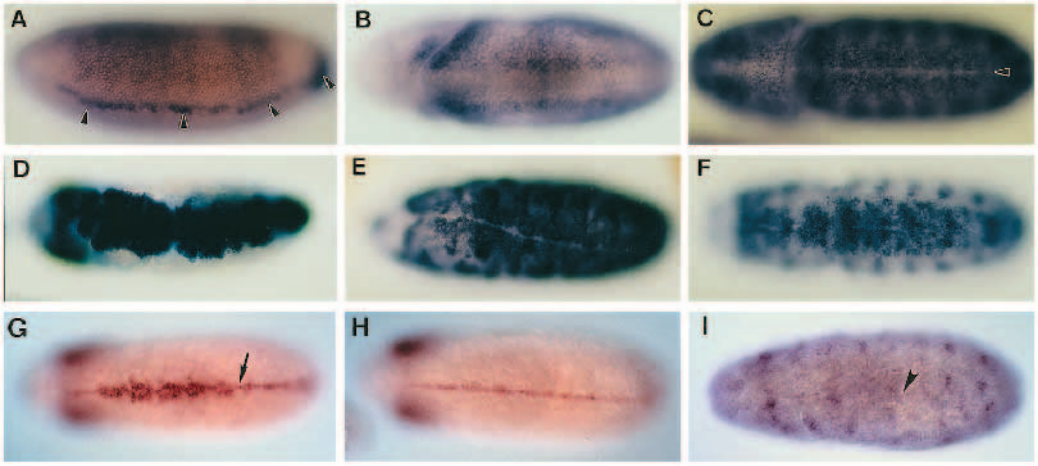

Fig. 5. Yan antagonizes the induction of ventralmost cell fates by Pointed. (A) A wild-type stage 5 embryo simultaneously hybridized with digoxigenin-labeled *yan* and *single minded* DNA probes. *singled minded* expression demarcates the presumptive midline cells (arrowheads). *yan* is first expressed in the cellular blastoderm in the neurogenic region and the dorsal ectoderm. Very low levels of *yan* expression were found in the dorsalmost blastoderm cells. *yan* expression is below the detection limit after 2-3 cell rows dorsal to the presumptive midline cells. (B) During gastrulation, *yan* expression is increased in the ventralmost ectodermal cells. (C) Expression in about 6-8 ventralmost ectodermal cell rows flanking the midline appears elevated compared to an otherwise more general expression pattern (stage 9/10). No *yan* expression is found in the midline cells (arrowhead). (D) Expression of *otd* in a stage 12 yan^{aopII5} embryo can be observed in a broad domain of up to eight cells rows flanking the ventral midline. In addition, the level of *otd* expression appears enhanced when compared to wild type (see Fig. 3A). (E) *aos* expression in a stage 11 yan^{aopII5} embryo. The domain of *aos* expression, as well as the level is increased. (F) *trn* expression in a stage 11 yan^{aopII5} embryo. Strong *trn* expression is found in up to eight cell rows flanking the ventral midline. (G) Induction of activated Yan by *Kr*-Gal4 (anterior border of *Kr* domain is shown by arrow); or *rho*-Gal4 (H) results in a dramatic reduction in the level of *otd* expression in the domain in which Yan was induced. (I) Similarly, expression of *aos* in the ventral domain (arrowhead) is abolished in *rho*-Gal4/UAS-activated Yan embryos. Note: *rho*-Gal4 drives the expression of target genes in the neuroectoderm.

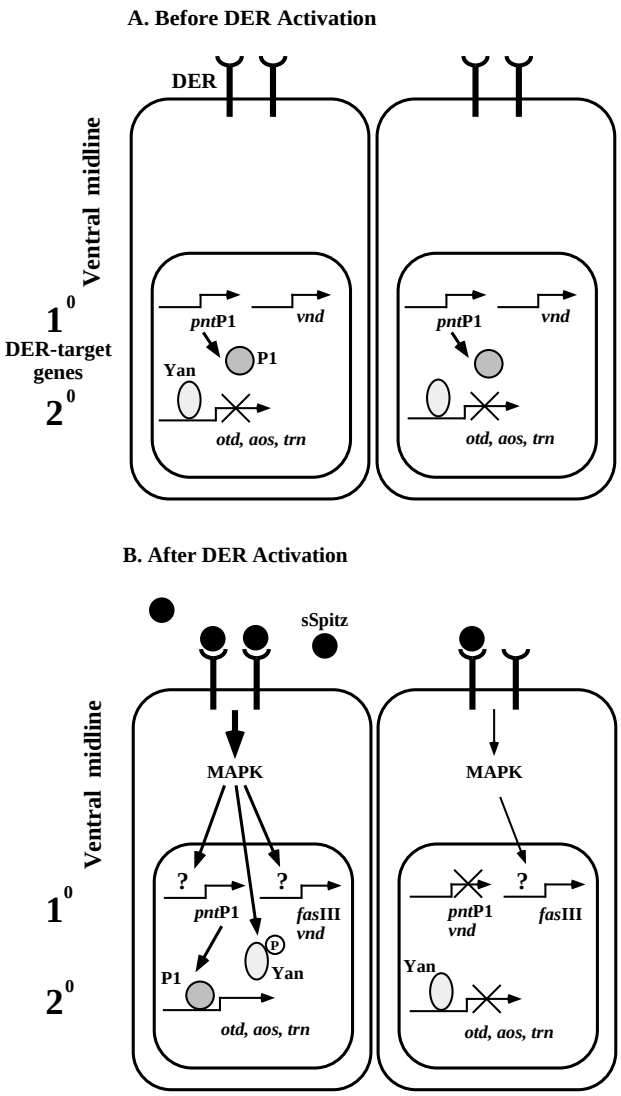

Fig. 6. A scheme for the induction of ventralmost cell fates by the DER pathway. (A) During cellular blastoderm, prior to DER activation, *pntP1*, *yan* and *vnd* are expressed in the ventral ectoderm, in a DER-independent manner. Binding of Yan to the promoters of the Pointed-target genes (e.g. *otd*, *argos* and *trn*), shuts off their transcription. (B) At the germband extended stage, DER is activated by secreted Spitz in a graded manner, such that the cells closest to the ventral midline exhibit the highest level of activation. In these cells, the DER pathway (presumably through MAP kinase), induces the transcription of *pntP1* (a primary DER-target gene), as well as efficient phosphorylation and subsequent inactivation of Yan. As a result, secondary DER-target genes (*otd*, *aos* and *trn*), are induced by Pointed P1. The expression of other primary DER-target genes, such as *vnd* and *fas*III, is also induced in the ventralmost cells. In more lateral cells, the level of DER activation is lower. Primary genes such as *pntP1* and *vnd* are not induced. Yan is not effectively phosphorylated, and thus blocks induction of secondary, Pointed-dependent target genes. Finally, other primary DER-target genes, which can be induced by lower levels of DER activation (e.g. *fas*III), are expressed in these cells.

tralmost cell fates by the DER pathway are emerging. The requirement for Yan inactivation may be a cooperative process. Eight MAP kinase phsophorylation sites were identified on Yan, but only one of them is absolutely essential for the normal response to the Ras/MAP kinase pathway (Rebay and Rubin, 1995). However, phosphorylation of the other sites may be required to modulate or amplify the response and could provide an additional factor in restricting the region where Yan inhibitory effects are efficiently eliminated by DER. The simultaneous requirement for the induction of *pntP1* transcription by DER should provide another spatial constraint. Concomitant activation of Pointed and inactivation of Yan was also demonstrated in the eye imaginal disc, where a combined response is achieved by MAP kinase phosphorylation of Pointed P2 and Yan (O'Neill et al., 1994; Brunner et al., 1994). It is interesting to note that, although the two forms of Pointed are affected in different ways by RTKs (transcriptional induction for *pointed P1* versus post-translational activation for Pointed P2), the end result is similar, in terms of activation of Pointed-target genes. However, how different target genes are activated in each tissue in response to Pointed activation, needs to be further analyzed.

Primary and secondary DER-target genes

The ventralmost markers *otd*, *argos* and *trn* respond to the nuclear levels of Pointed P1 and Yan. Since *pntP1* is induced by DER, they represent the secondary-target genes responding to DER activation. In contrast, the *vnd* and *fas*III genes, on the contrary, may represent additional primary DER-target genes. Their expression is altered in *DER* mutant embryos or following hyperactivation of DER, but they do not respond to modifications in Pointed P1 levels. Whereas induction of *pntP1* is required for the generation of ventral ectodermal structures, induction of *vnd* by DER may influence the specification of ventral neuroblasts.

Among the candidate primary-target genes, *pntP1* and *vnd* are induced in the ventralmost cells, while *fas*III is expressed also in the ventrolateral cell rows. It is not known which molecules mediate the transcriptional activation of the primary DER-target genes. A possible candidate is the Jun protein, which is ubiquitously expressed in the embryo (Zhang et al., 1990), and can be phosphorylated and activated by receptor tyrosine kinase pathways. An attractive possibility is that the same molecules are involved in triggering the ventralmost and the ventrolateral target genes, and differences in affinities to the promoter sites would dictate the expression borders of each gene. A model for the transcriptional responses to DER activation in the ventral ectoderm is presented in Fig. 6.

pntP1 and *yan* are expressed in additional tissues in which DER activation takes place. In the ovary, *yan* is expressed in both anterior and posterior follicle cells. In the posterior follicle cells, DER-dependent induction of *pntP1* transcription takes place (Gabay and Shilo, unpublished data). The accumulation of Pointed in the follicle cells, in conjunction with the DER-dependent inactivation of Yan, could provide the binary switch, triggering differentiation to the posterior fate and subsequent signaling into the oocyte by these cells. The identification of *pntP1* and Yan as downstream targets of the DER pathway may thus represent a general paradigm for different developmental decisions in which the DER pathway is involved.

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