Development 128, 4837-4846 (2001)
Printed in Great Britain © The Company of Biologists Limited 2001
DEV7914

The functions of *pannier* during *Drosophila* embryogenesis

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Accepted 7 September 2001

SUMMARY

The *pannier* (*pnr*) gene of *Drosophila* encodes a zinc-finger transcription factor of the GATA family and is involved in several developmental processes during embryonic and imaginal development. We report some novel aspects of the regulation and function of *pnr* during embryogenesis. Previous work has shown that *pnr* is activated by decapentaplegic (*dpp*) in early development, but we find that after stage 10, the roles are reversed and *pnr* becomes an upstream regulator of *dpp*. This function of *pnr* is necessary for the activation of the Dpp pathway in the epidermal cells implicated in dorsal closure and is not mediated by the JNK pathway, which is also necessary for Dpp activity in these cells. In addition, we show that *pnr* behaves as a selector-like gene in generating morphological diversity in the dorsoventral body axis. It is responsible for maintaining a subdivision of the dorsal half of the embryo into two distinct, dorsomedial and dorsolateral, regions, and also specifies the identity of the dorsomedial region. These results, together with prior work on its function in adults, suggest that *pnr* is a major factor in the genetic subdivision of the body of *Drosophila*.

Key words: Pnr, GATA factors, Selector genes, DV body axis, Dpp, Dorsal closure, *Drosophila*

INTRODUCTION

The morphological diversity in *Drosophila* is primarily established along the anteroposterior (AP) and the dorsoventral (DV) body axes. A great deal is known about the genetic factors that generate the diversity along the AP axis (Lawrence, 1992). Maternal products such as Bicoid and Caudal form functional gradients, which are resolved in the activation of a cascade of zygotic (gap, pair-rule, polarity) genes (Nüsslein-Volhard and Wieschaus, 1980). The end product of this process is the formation of a chain of 14 metameric units (parasegments) (Martinez-Arias and Lawrence, 1985), each composed of two stripes of cells, one expressing the gene *engrailed* (*en*) and the other not. The morphological diversity is then generated by the various Hox genes, which become active in different sets of parasegments (Lawrence and Morata, 1994; Mann and Morata, 2000).

By contrast, relatively little is known about genetic subdivisions of the body in the DV axis. A crucial event is the formation in early embryogenesis of a gradient of nuclear expression of the Dorsal protein, whose nuclear translocation requires the activity of the Toll receptor. Spatial restriction of Toll activity is dependent on the accumulation in the ventral region of the active form of the Toll ligand Spatzle, the result of a proteolytic processing catalysed by the serine protease encoded by the gene *easter*. In turn, the restriction of Easter activity to the ventral region depends on the localised activity of the heparan sulfate transferase encoded by *pipe*. The Pipe protein is thought to modify the proteoglycans of the matrix to allow interaction with the Easter protease in order to cleave the Spatzle protein (Anderson, 1998).

The Dorsal gradient is a principal element establishing local differences along the DV axis. Different levels of nuclear Dorsal regulate the activity of zygotic target genes such as *snail*, *rhomboid* and *decapentaplegic* (*dpp*), which are involved in the specification of different cell types. In early embryos, the Dpp product is localised in the dorsal half and its activity determines the formation of dorsal embryonic structures: in absence of *dpp* activity embryos become ventralised (Irish and Gelbart, 1987), whereas derepressed activity of the Dpp pathway results in dorsalised embryos (Nellen et al., 1996).

The subdivision of the dorsal ectoderm into distinct parts is achieved through the establishment of a complex Dpp activity gradient in the early embryo. This involves the function of another TGFβ molecule, encoded by *Screw* (*Scw*), which potentiates Dpp signalling (Arora et al., 1994), and that of the secreted protein Short gastrulation (Sog). The activity of Dpp/Scw is modulated by the Sog gradient (Neul and Ferguson, 1998): high Sog levels in the lateral region block Dpp/Scw and allow the formation of neuroectoderm; intermediate levels attenuate Dpp/Scw function to specify dorsal epidermis; low Sog levels enhance Dpp/Scw activity to form the most dorsal tissue, amnioserosa (Ashe and Levine, 1999). The response to Dpp/Scw is further complicated by the activity of *brinker* (*brk*), which encodes a transcriptional repressor (Zhang et al., 2001) and is expressed in lateral stripes in the neuroectoderm (Jazwinska et al., 1999b). *brk* suppresses the response to Dpp signalling, but its activity is repressed by high levels of Dpp in the dorsal ectoderm (Jazwinska et al., 1999b). The nature of the interactions between Dpp and Brk draws a border of the patterning influence of Dpp/Scw.

In addition to its early role specifying dorsal ectoderm, *dpp*

has other embryonic functions that are independent of the polarity of the DV axis; it plays a role in dorsal closure, midgut development and tracheal formation (Affolter et al., 1994). The existence of these other functions is reflected in the dynamics of its expression. Although in early development *dpp* transcripts cover the half dorsal region of the embryo, after stage 11 (germ band elongation) they disappear from much of the dorsal embryos and become restricted to two longitudinal stripes: a dorsal one at the border of the epidermis with the amnioserosa, and the other in the lateral region (St Johnston and Gelbart, 1987). The expression of *dpp* in the dorsal stripe is under the control of the JNK pathway (Glise and Noselli, 1997) and is involved in embryonic dorsal closure.

The Dpp activity gradient probably achieves its different roles by activating various target genes. Several of these have been identified that respond to different thresholds of the Dpp/Scw gradient (Ashe et al., 2000). They include *Race*, *hindsight*, *tailup*, *u-shaped* (*ush*) and *pannier* (*pnr*) (Ramain et al., 1993; Frank and Rushlow, 1996; Rush and Levine, 1997), which define distinct dorsal domains and are probably instrumental in subdividing the dorsal ectoderm into different parts. *pnr* is a gene encoding a zinc-finger protein containing a GATA motif (Ramain et al., 1993; Winick et al., 1993), which has several embryonic functions connected with dorsal closure and heart development (Heitzler et al., 1996; Gajewski et al., 1999).

During embryogenesis, *pnr* is expressed in a complex pattern (Winick et al., 1993; Heitzler et al., 1996); in early embryos it is expressed in a broad dorsal domain extending from 20% to 60% of the egg length (Winick et al., 1993), a region including the presumptive amnioserosa and dorsal epidermis. This pattern is later refined, and by germ band retraction *pnr* is expressed in a longitudinal dorsal domain extending along the thoracic and abdominal segments (Calleja et al., 2000). This late embryonic pattern resembles that described for imaginal development, where it has been shown that *pnr* has an instructive, selector-like function, determining the identity of the medial dorsal structures of thoracic and abdominal segments (Calleja et al., 2000).

pnr embryonic expression and its role in adult development suggest that it may be involved in subdividing the dorsal part of the body into distinct genetic domains, but to date this possibility has not been examined. It has been reported that in *pnr* mutant embryos dorsal closure is defective and as a consequence the embryos present 'holes' in the dorsal cuticle (Heitzler et al., 1996). We investigate the embryonic function of *pnr* by studying the effects of alterations of *pnr* activity on the larval patterns and on the expression of genes involved in larval patterning. We show that it has an instructive role in specifying the dorsomedial pattern of all thoracic and abdominal segments. Our results indicate that *pnr* is the gene responsible for a major subdivision along the DV axis in the *Drosophila* body. We also show that *pnr* is involved in embryonic dorsal closure by activating *dpp* in the cells in the leading edge.

MATERIALS AND METHODS

Drosophila stocks

The pnr^{VX6} allele has been described previously (Heitzler et al., 1996), and can be regarded as a null allele, as most of the coding sequence (except that coding for nine amino acids) is lacking. In addition we used the following mutants all which are considered null alleles: $Abd\text{-}M^{M1}$ (Casanova et al., 1986); $grain^{7L12}$ (Brown and Castelli-Gair Hombria, 2000); *Df(2L)5* (deficient for *sal* and *sal-r*) (de Celis et al., 1996); ems^{9Q64} (Dalton et al., 1989); brk^{M68} (Jazwinska et al., 1999a); $Df(3)iro^{2}$ (Leyns et al., 1996); and lin^{G1} (Bokor and DiNardo, 1996).

To distinguish hemizygous or homozygous mutant embryos from their heterozygous siblings, we made use of balancer chromosomes carrying *lacZ* transgenes: *FM7c ftz-lacZ* (Klambt et al., 1991), *CyO wg-lacZ* (Ingham et al., 1991) and *TM3 hb lacZ* (Hyduk and Percival-Smith, 1996). Other *lacZ* lines used were *en-lacZ* (Simcox et al., 1991), brk^{M12}*-lacZ* (Jazwinska et al., 1999a) and *ush-lacZ.*

Gal4/UAS experiments

The *UAS-pnr* chromosome was a gift from Mariann Bienz and has bee described elsewhere (Heitzler et al., 1996). We also used the $UAS\text{-}tkv^{DN}$ (Haerry et al., 1998). The Gal4 lines used were *en-Gal4* (Tabata et al., 1995), *Ubx-Gal4* (M. Calleja and G. M., unpublished), *arm-Gal4* (Sanson et al., 1996), *ptc-Gal4* (Wilder and Perrimon, 1995) and *wg-Gal4* (M. Calleja and G. M., unpublished). *LP1-Gal4* drives expression in the amnioserosa (G. M., unpublished).

Analysis of embryonic cuticles

Embryos were collected overnight and aged an additional 12 hours, then first instar larvae were dechorionated in commercial bleach for 3 minutes and the vitelline membrane removed using heptano-methanol 1:1. Then, after washing with methanol and 0.1% Triton X-100, larvae were mounted in Hoyer's lactic acid 1:1 and allow to clear at 65°C for at least 24 hours.

Immunostaining

Embryos were stained using standard procedures for confocal microscopy (Gonzalez-Crespo et al., 1998); secondary antibodies were coupled to Red-X and FITC fluorochroms (Jackson Immunoresearch) and embryos were analysed under a laser-scan Zeiss microscope.

In situ hybridisation and antibody/in situ hybridisation double labelling were performed as described previously (Azpiazu and Frasch, 1993), and embryos were mounted in Permount (Fisher Scientific). Digoxigenin-labelled RNA probes were synthesised as described (Tautz and Pfeifle, 1989). Those used were pnr full-length antisense RNA probe synthesised from a plasmid provided by Pat Simpson and dpp antisense RNA probe synthesised from a plasmid provided by Ana Macías.

The antibodies used were anti-Cad (Macdonald and Struhl, 1986), anti BP102 (hybridoma bank), anti-Eve (Frasch et al., 1986), anti-Ftz (Krause et al., 1988), anti-Kr (provided by Jordi Casanova), anti-Pnr and rabbit anti-β-galactosidase (Capel).

Production of an anti-PNR antibody

We have studied (with the help of Juan Pablo Albar of the Centro Nacional de Biotecnologia) the amino-acid sequence of the Pnr protein using the 'PeptieStructure' program, which makes secondary structure predictions for a peptide sequence. The predictions include measures for antigenicity index, chain flexibility, hydrophobicity and surface probability. In accordance with these data, we have chosen two peptides: a first peptide spanning amino acids 7 to 26 (DGDSTSDQQSTRDYPHFSGDY**C**) and a second from amino acid 272 to 284 (TRKRKPKKTGSGS**C**). The peptides were prepared as a fusion with KLH to increase the antigenicity of each peptide. Antiserum against these peptides was raised in rabbits. We performed the first injection with 250 μg of a mix of the two peptides and the next five injections with a mix of 125 μg each injection. The second injection was 21 days after the first, and the other boosters were given also with a 21 days interval. Antiserum from the rabbit was tested against fixed *Drosophila* embryos.

RESULTS

Expression and regulation of *pnr* during embryogenesis

The embryonic expression of *pnr* has already been described (Winick et al., 1993; Heitzler et al., 1996; Calleja et al., 2000), therefore it will only be considered briefly here. We have assayed the distribution of Pnr products by in situ hybridisation using an RNA probe and also using an anti-Pnr antibody made in our laboratory (see Materials and Methods). As expected for a protein containing DNA-binding motifs, the Pnr product appears localised to the cell nuclei (Fig. 1). We found a good correlation between the patterns of RNA and protein distribution after embryonic stage 7, when the Pnr protein is first detected with the antibody.

In early embryos, *pnr* is expressed in a broad region on the dorsal side, which may occupy as much as 40% of the circumference of the embryo. It does not extend to the entire length of the embryo. The anterior and posterior borders of expression can be delimited by double staining of *pnr* with *even-skipped* (*eve*), *fuzhi tarazu* (*ftz*), *caudal* (*cad*) and *engrailed* (*en*) (Fig. 1). The anterior border is slightly anterior to the second eve stripe, which corresponds to parasegment 3 (Labp-T1a), whereas the posterior border abuts the 7th ftz stripe, which marks the anterior limit of parasegment 14 (Lawrence, 1992). Thus, the *pnr* embryonic domain extends from the labial to the ninth abdominal (A9) segment: the presumptive region of part of the head and the entire thorax and abdomen of larvae and adult flies.

As development proceeds the overall extent of the pnr domain in the AP axis does not change; the only significant modification is that between embryonic stages 10 and 11, *pnr* transcription is repressed in much of the A8 segment (Fig. 1B,D), thus leaving a gap of expression that has already been noted (Calleja et al., 2000). The small posterior portion in the posterior region in late embryos that does not contains *pnr* activity exhibits *caudal* (*cad*) activity (Fig. 1E). It corresponds to the presumptive A10 segment (Moreno and Morata, 1999), which gives rise to analia structures.

The extent of the Pnr domain in the DV axis is also modified during embryogenesis; by stage 10 there is no detectable *pnr* activity in the amnioserosa cells (Fig. 1B), even though in earlier stages it is expressed in the amnioserosa presumptive region (Fig. 1A,C). We have mapped *pnr* expression (Fig. 1F) with respect to that of *dpp*: a determinant of dorsal development in embryos and a positive regulator of *pnr* (Winick et al., 1993). In early embryonic stages, *dpp* is expressed in the dorsal half of the embryo (Ferguson and Anderson, 1992), but by stage 10 the Dpp product lacks in the most dorsal tissue (amnioserosa) and occupies about half of the epidermis, from the border of the amnioserosa to a mid-lateral region (St Johnston and Gelbart, 1987). This is later resolved in two stripes by subsequent loss of expression in the mid-dorsal region. The expression of *pnr* is confined within the domain defined by the two dpp stripes. *dpp* and *pnr* overlap in the dorsal region, and share a common border with the amnioserosa (Fig. 1F).

We have studied some aspects of the regulation of *pnr* activity. The loss of expression in the A8 segment depends on *Abdominal-B* (*Abd-B*) activity: in *Abd-B* mutants *pnr* is expressed in the A8 site (Fig. 2A). However, none of the known *Abd-B* target genes expressed in the A8 segment, *spalt* (*sal*),

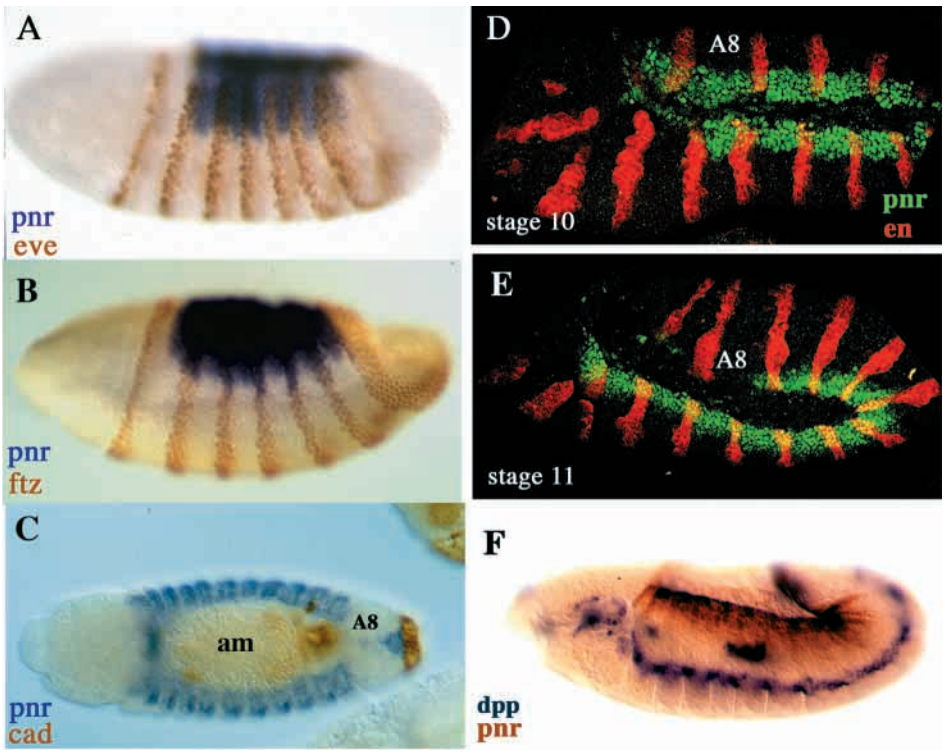

Fig. 1. Expression of *pnr* during embryonic development. (A,B) Lateral view (anterior left, dorsal up) of early embryos (stages 5-6) showing the distribution of *pnr* transcripts, and Eve (A) and Ftz (B) proteins. *pnr* expression covers a broad dorsal domain. The anterior limit is close to the second eve stripe, although there is some low level expression anterior to the eve stripe, which is not visible in the picture. The posterior limit coincides well with the anterior border of the seventh Ftz stripe. (C) Dorsal view of a late embryo (stage 13) doubly labelled for *pnr* mRNA and Cad protein. *pnr* transcripts lack in the amnioserosa region (am) and in the A8 segment. There is *pnr* expression in A9, but not in A10, where *cad* is expressed. (D,E) Confocal images of lateral view of *en-lacZ* embryos doubly stained with anti-Pnr antibody and anti β-gal. The spotty appearance of Pnr label indicates the protein is in the cell nucleus. There is Pnr protein in the A8 segment in stage 10 (D), but not in stage 11 (E). (F) Lateral view of a stage 12 embryo showing *pnr* and *dpp* expression. The dorsal dpp stripe is within the Pnr domain and shares the same dorsal limit at the junction with the amnioserosa.

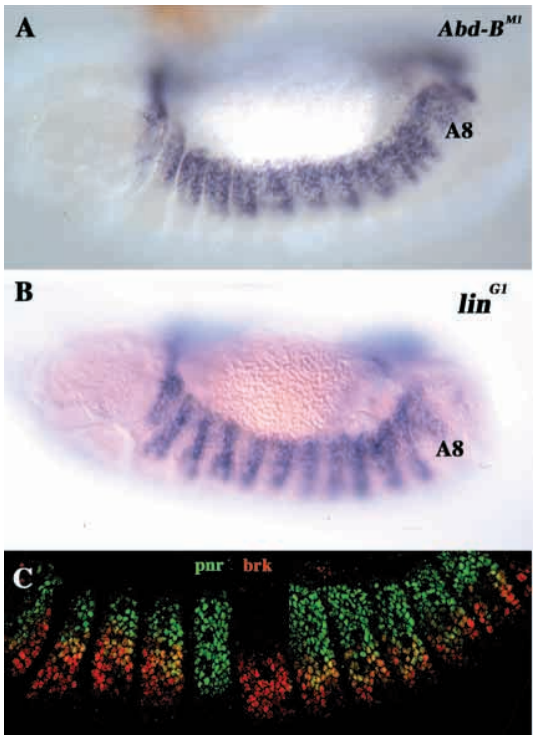

Fig. 2. (A,B) Distribution of pnr RNA in mutant embryos for *Abd-B*^{M1} and *lin*^{G1}. There is *pnr* expression in A8, in contrast to wild-type embryos (compare with Fig. 1C). (C) Confocal images of a double labelling for *pnr* and *brk* expression in several segments of an stage 13 embryo. Dorsal is towards the top. Although *brk* (red) is expressed ventral to *pnr* (green) there is a zone of overlap, as indicated by comparing the images of the A1 and the A2 segments, for which only the green (A1) and red (A2) channels are shown.

empty spiracles (*ems*) or *grain* (*gnr*) (Castelli-Gair, 1998), mediates this regulation, because *pnr* expression is not altered after mutation of any of these genes (not shown). Finally, we found that in mutant embryos for *lines* (*lin*), a co-factor of *Abd-B* function (Castelli-Gair, 1998), *pnr* is not downregulated in A8 (Fig. 2B).

As mentioned above, *pnr* expression is switched off in the amnioserosa region before germ band extension. The dorsal limit of *pnr* expression coincides with the morphological boundary between dorsal epidermis and the amnioserosa (Fig. 1C) and abuts the expression domain of *Kruppel* (*Kr*), which at that time is expressed in all amnioserosa cells. We do not know the identity of the factor(s) that suppress *pnr* transcription in the amnioserosa, although we have observed that there is no alteration of *pnr* expression in *Kr* mutants (not shown). Several amnioserosa-specific genes [*Race*, *zen*, *tail-up*, *hindsight* and *serpent* (Frank and Rushlow, 1996)] are candidates for this regulation.

On the ventral side, the pnr domain abuts that of *iro* (Calleja et al., 2000), raising the possibility that *iro* might be a negative regulator of *pnr*. However homozygous *Df(3L)iro*^{2} embryos, totally deficient for the Iroquois complex (Leyns et al., 1996), show normal *pnr* expression (not shown).

Because *pnr* is activated by *dpp* in early development (Winick et al., 1993; Ashe et al., 2000), we have checked whether its late expression is negatively regulated by *brk*, an antagonist of the Dpp pathway (Campbell and Tomlinson, 1999; Jazwinska, et al., 1999a; Minami et al., 1999). *brk* is expressed in a longitudinal domain in the lateral region of the embryo (Jazwinska et al., 1999b), close to the Pnr domain; thus, it might regulate a possible activating role of *dpp* on *pnr*. Besides, there is evidence (Jazwinska et al., 1999b; Ashe et al., 2000) that alterations in *brk* activity modify the extent of the early Pnr domain. Double label experiments show that in wild-type late embryos (from stage 10), *pnr* and *brk* define parallel longitudinal domains. *brk* is expressed in a more ventral position but there is an ample zone of overlap between the two domains (Fig. 2C). In *brk*^{M68} embryos, *pnr* expression from stage 10 onwards is like the wild type (Marty et al., 2000). As there is compelling evidence that early *pnr* activity is regulated by *brk* levels (Jazwinska et al., 1999b; Ashe et al., 2000), it

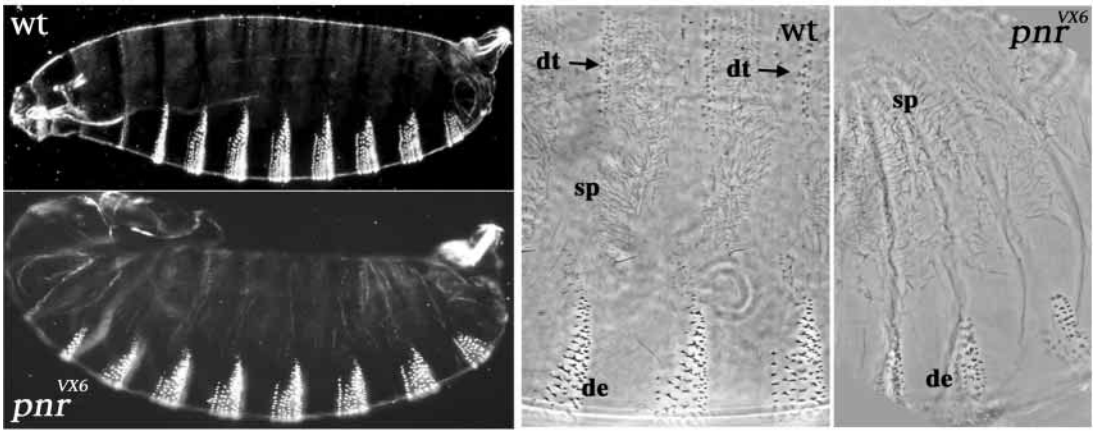

Fig. 3. Larval phenotype of *pnr*^{VX6} larvae. The two dark-field photographs on the left show a lateral view of a wild-type and a *pnr*^{VX6} first instar larva and the phase contrast photographs on the right compare epidermal pattern elements of the two genotypes. The *pnr*^{VX6} exhibits the characteristic basket shape, and the dorsal closure is defective (not visible in the photo). From a side view it is possible to recognise three different pattern elements arrange along the DV axis of the wild-type. In the most dorsal position (top) there are dorsal triangles (dt, arrows) and spinules (sp), but in the dorsolateral region there are only spinules. In the ventral side of the larva (bottom), the principal elements are the denticles (de). Spinules and dorsal triangles are lacking in this region. In *pnr*^{VX6} larvae, the dorsal triangles are missing but spinules form and appear to be expanded towards the dorsal region.

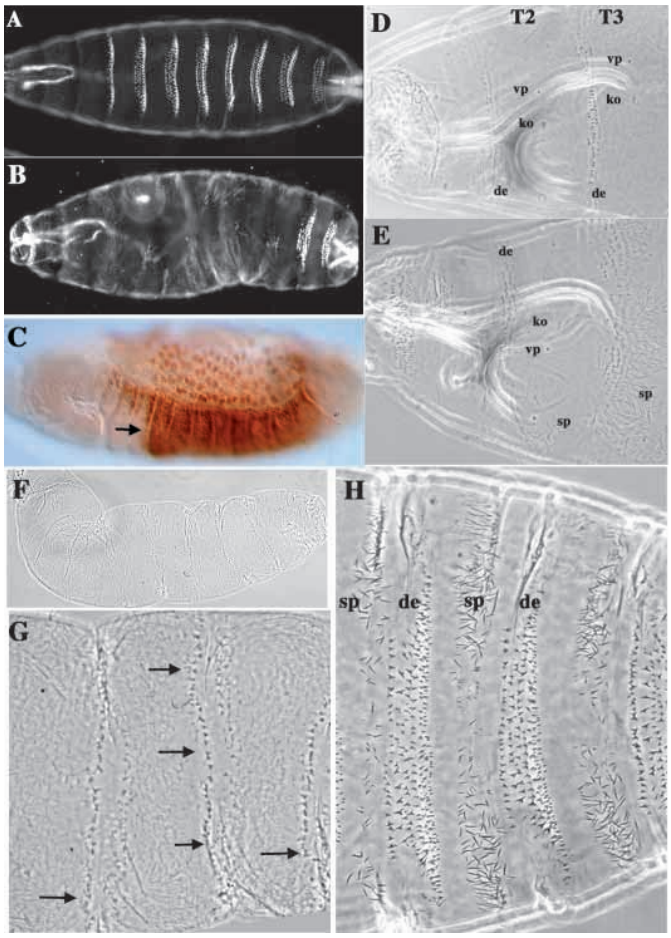

Fig. 4. Developmental consequences of ectopic *pnr* activity. (A) Ventral view of a wild-type first instar larva showing the characteristic denticle belts that differentiate in the ventral side. (B) Ventral view of a first instar larva of genotype *Ubx-Gal4/UAS-pnr* showing transformation of the ventral region into dorsal one. Note that the ventral denticles of most of the abdomen are replaced by dorsal spinules which are thinner. (C) Embryo of the same genotype as in B stained with anti-Pnr antibody to show that the Pnr protein is present in the Ubx domain. The area stained covers the sum of the normal domains of *pnr* and *Ubx*. The arrow marks the anterior limit of the Ubx domain (T2p); from this point the Pnr protein is present in high levels down to abdominal segment 6. Note *pnr* expression in the amnioserosa cells. (D,E) Phase contrast pictures of the thoracic region of a wild-type and a *Ubx-Gal4/UAS-pnr* larva. In the anterior region of the T2 segment there is no difference between them; they differentiate typical ventral thoracic pattern elements such as Keilin's organs (ko), ventral pits (vp) and denticles (de). In the posterior T2 segment, the *Ubx-Gal4/UAS-pnr* larva exhibits some spinules (sp), characteristic dorsal elements, but the differences are clearer in T3 where all ventral elements lack and are replaced by spinules. (F,G) Ventral view of an *arm-Gal4/UAS-pnr* larva showing a virtually complete transformation of ventral into dorsal structures. Three abdominal segments are magnified in G to show (arrows) the presence of dorsal triangles around the circumference of the larva, thus suggesting the transformation is towards dorsomedial pattern. (H) Ventral view of abdominal segments of a larva of genotype *wg-Gal4/UAS-pnr*. As *wg* is expressed in the region normally differentiating naked cuticle, the denticle (de) belts are not affected, but part of the naked region is transformed into the corresponding dorsal one and differentiates dorsal spinules (sp).

suggests that *pnr* is under different control in late embryonic development. This is supported by the observation that in embryos lacking *tkv* zygotic function, the extent of the pnr domain is normal, although expression levels are weaker than in wild-type embryos (Affolter et al., 1994). It also supported by our finding that driving a dominant negative form of *thick veins* (*UAS-tkv-DN*) (Haerry et al., 1998) with *Ubx-Gal4* does not alter normal *pnr* activity (not shown).

The developmental role of *pnr* during embryogenesis: phenotype of loss and of gain of *pnr* activity

We have studied the effects on the larval cuticle patterns of alterations in *pnr* activity. The principal morphological features of the dorsal and ventral epidermis of the wild-type first instar larva are illustrated in Fig. 3. There are various types of cuticle differentiations on the dorsal side, which are easily discernible from the thick denticles present in the ventral side. The arrangement of cell types is not uniform in the dorsal cuticle. The dorsomedial region differentiates all the dorsal pattern elements, described by Heemskerk and DiNardo (Heemskerk and DiNardo, 1994), but the dorsolateral region lacks some of these elements. Especially relevant is the lack of dorsal triangles [cell type 1 in Heemskerk and DiNardo (Heemskerk and DiNardo, 1994)] in the dorsolateral region (Fig. 4), which differentiates only spinules (cell type 4). These dorsal triangles are especially clear in the abdominal segments. As they do not extend to the lateral region, the distinction between the medial and lateral region of the dorsal epidermis can be assayed by the presence or absence of dorsal triangles.

Larval phenotype of the pnr^{VX6} mutation

For the description of the null phenotype of *pnr*, we have used the pnr^{VX6} mutation, which has been characterised genetically and molecularly (Ramain et al., 1993; Heitzler et al., 1996). It is a small deletion that removes all but nine amino acids of the Pnr protein (Ramain et al., 1993); it can therefore be considered as a null mutation. Homozygous pnr^{VX6} embryos show no staining with anti-Pnr antibody.

There are two principal phenotypic alterations in pnr^{VX6} embryos. The first is that dorsal closure is defective, as has already been reported (Heitzler et al., 1996). The left and right sides do not fuse properly, often leaving 'holes' in the dorsal cuticle, which gives the embryos a characteristic basket shape. This indicates an involvement on *pnr* in dorsal closure that we examine below. Although there are holes in the dorsal epidermis, dorsal cuticular elements are present in pnr^{VX6} larvae (Fig. 3).

The second phenotypic trait is the disappearance in the abdominal region of the most dorsal pattern elements, the dorsal triangles, which appear to be replaced by dorsolateral spinules (Fig. 3). Our interpretation is that in the absence of *pnr* the dorsomedial pattern cannot be formed and the dorsolateral pattern extends dorsally. We have measured the width of the dorsal domain (as indicated by the distance from the border of the amnioserosa to the middle Dpp stripe) and found that there is a normal number of cells. This suggests that in the absence of *pnr* function, there is no cell loss but that the dorsomedial domain is transformed into the dorsolateral one. This is in good agreement with the previous observation (Calleja et al., 2000) that in pnr^{VX6} mutant embryos the iro

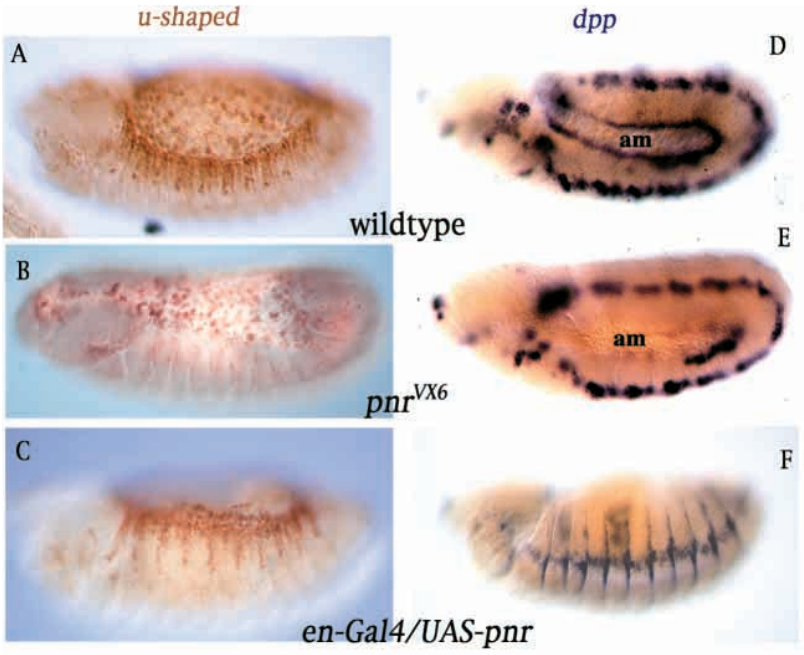

Fig. 5. *pnr* acts as a positive regulator of *ush* and *dpp* in late embryonic development. (A) Wild-type stage 13 embryo showing *ush* expression. It covers the amnioserosa and an epidermal region where it is coincident with *pnr*. (B) *ush* expression in pnr^{VX6} embryo. The epidermal expression has disappeared but it remains in the amnioserosa. (C) *ush* expression in an *en-Gal4/UAS-pnr* embryos showing ectopic *ush* activity in the posterior compartments. (D) Wild-type expression of *dpp* in an stage 11 embryo. There are two parallel stripes of *dpp* expression extending from the head to the end of the abdomen; the dorsal one abuts the amnioserosa (am). (E) *dpp* expression in a pnr^{VX6} embryo showing the lack of the dorsal dpp stripe close to the amnioserosa, whereas the lateral one is not affected. (F) *en-Gal4/UAS-pnr* stage 13 embryo showing ectopic *dpp* activity in the posterior compartments dorsal and ventral to the lateral dpp stripe. The dorsal stripe is not visible in the picture.

domain extends dorsally. As expected, no effect is seen in the ventral body region.

We expected an effect on the amnioserosa, because *pnr* is expressed in early embryos in the entire dorsal half, which includes the presumptive amnioserosa region. Moreover, Heizler et al. (Heizler et al., 1996) report that in pnr^{VX6} mutants, amnioserosa cells die prematurely. However, we fail to see any alteration in pnr^{VX6} embryos; the amnioserosa cells appear morphologically normal until the end of embryogenesis. They also express molecular markers such as *ush* (Fig. 5B). Besides, a characteristic phenotypic trait of the genes required for amnioserosa development is that the mutant embryos adopt an u-shaped morphology (Frank and Rushlow, 1996), owing to their inability to retract the germ band. In pnr^{VX6} embryos, germ band retraction is normal, suggesting that amnioserosa development is not affected.

Ectopic expression of *pnr*

We have used the Gal4/UAS method (Brand and Perrimon, 1993) to study the developmental potential of the Pnr protein during embryogenesis. Some Gal4 lines drive generalised expression (*arm-Gal4*) and others are restricted to different body parts (*Ubx-Gal4, ptc-Gal4, wg-Gal4, en-Gal4, LP1-Gal4*). We first observed that increased levels of the Pnr product (as in *pnr-Gal4/UAS-pnr*) do not have a detectable effect on larval patterns. This was expected because flies of *pnr-Gal4/UAS-pnr* genotype survive and show virtually wild-type phenotype (M. Calleja and G. M., unpublished).

The principal conclusion from the ectopic expression experiments can be summarised by saying that *pnr* is able to induce a transformation of the ventral and dorsolateral patterns into the mediodorsal ones. In *arm-Gal4/UAS-pnr* larvae, the entire epidermis becomes dorsalised (Fig. 4F,G). Close inspection of these larvae shows the presence in the abdominal segments of a continuous belt of dorsal triangles, indicating that the transformation is towards the mediodorsal pattern. A similar observation is made using a *Ubx-Gal4* driver (Fig. 4B,E). This line mimics the expression of the wild-type *Ultrabithorax* (*Ubx*) gene in embryos and shows expression from the posterior compartment of the second thoracic segment (T2) down to the abdominal segment A7, although it is weaker in the more posterior abdominal segments. The presence of the Pnr protein in the entire Ubx domain can be demonstrated with the anti-Pnr antibody (Fig. 4C).

The transformation of ventral to dorsal epidermis can also be seen in lines driving expression in restricted regions of segments. *en-Gal4/UAS-pnr* embryos show the transformation in the P compartments; a thin stripe of dorsal epidermis can be seen in the ventral region of each segment. In *wg-Gal4/UAS-pnr* embryos the transformation is restricted to a portion of the anterior compartment (Fig. 4H) that corresponds to the embryonic expression of *wg*, just anterior to the en stripe (Bejsovec and Martinez Arias, 1991). These results suggest that transformation induced by Pnr is cell autonomous, restricted only to the cells containing the product.

We have not tested whether the effect of ectopic *pnr* expression extends to the mesoderm, but it clearly affects the central nervous system (CNS). In *Ubx-Gal4/UAS-pnr* embryos, the ventral cord is clearly altered, precisely in the Ubx domain (not shown), suggesting that the transformation induced by *pnr* affects all the ectodermal derivatives.

In contrast to the observed in the epidermis and the CNS, ectopic *pnr* expression does not seem to affect in the amnioserosa, the most dorsal ectodermal derivative. In *Ubx-Gal4/UAS-pnr* embryos the amnioserosa develops normally even though it contains high levels of Pnr protein (Fig. 4C). We have used a amnioserosa specific driver LP1 (see Fig. 7) to express *pnr* only in this tissue and do not observe any defect. In *Ubx-Gal4/UAS-pnr* or *LP1/UAS-pnr* embryos, germ band retraction is normal.

Regulatory roles of *pnr*

We have analysed the regulatory interactions of *pnr* with *ush* and *dpp*, whose expression domains overlap with that of *pnr*. The negative control role of *pnr* on *iro* activity has already been reported (Calleja et al., 2000).

The wild-type expression of *ush* is shown in Fig. 5A; it covers the amnioserosa and also part of the dorsal domain in

the epidermis, where it overlaps with *pnr*. In the dorsal epidermis, the ush domain is similar to that of *pnr*: the two genes define longitudinal domains and both are downregulated in A8. The difference is that the ush domain is narrower. In absence of *pnr* activity (*pnr*^{VX6} embryos), *ush* expression in the epidermis is abolished, whereas that in the amnioserosa it is unaffected (Fig. 5B). Conversely, ectopic *pnr* activity induces *ush* expression outside its normal domain (Fig. 5C), suggesting an upstream control by *pnr*. This control of *ush* by *pnr* provides an explanation for the downregulation of *ush* in the A8 segment: *ush* expression depends on that of *pnr*, which is turned off. We note that *ush* has to have other regulators, because its expression in the amnioserosa does not depend on *pnr* (Fig. 5B).

The wild-type expression of *dpp* changes during embryogenesis, suggesting the existence of several regulatory tiers; the original broad dorsal expression is resolved in late embryonic stages into two thin stripes running in the anteroposterior direction (Fig. 5D). A dorsal stripe is located at the junction of the epidermis with the amnioserosa, whereas the other is located more ventrally. The dorsal stripe probably reflects a requirement for activity of the Dpp pathway in dorsal closure, as indicated by the dorsal open phenotype of mutations in Dpp transducers such as *think veins* and *punt* (Affolter et al., 1994). It is under the control of the JNK pathway (Glise and Noselli, 1997). It requires the activity of *hemipterous* (*hep*) a mitogen-activated protein kinase kinase (MAPKK) related to vertebrate Jun N-terminal kinase kinase (JNKK). *hep* controls dorsal closure by independently activating *dpp* and *puckered* (*pc*) a gene necessary for the movement of the leading edges during dorsal closure (Martin-Blanco et al., 1998).

We find that, just as in *hep* mutants, in *pnr*^{VX6} embryos, the dorsal Dpp stripe disappears, although the stripe located more ventrally is not altered (Fig. 5E). Moreover, ectopic *pnr* activity also induces ectopic *dpp* expression (Fig. 5F). These results argue that *pnr* acts as a positive regulator of *dpp* in late embryogenesis. We note, however, that the dorsal dpp stripe of the wild type is not interrupted in A8, as might be expected if it required continuous *pnr* activity. As the downregulation of *pnr* occurs between stages 10 and 11 (Fig. 1B,D) and by that time the dorsal dpp stripe is already formed, we suspect the earlier *pnr* expression induces *dpp* activity in A8 and later *dpp* maintains its own expression.

The loss of the dorsal dpp stripe in the absence of either *pnr* function or JNK activity (Glise and Noselli, 1997) suggested that *pnr* might be required for the initiation or functioning of the JNK pathway. Therefore, we checked the activity of the JNK pathway in *pnr*^{VX6} embryos by examining the expression of *puc*, the final element of the cascade. The result is that *puc* activity is not altered (Fig. 6), indicating that the formation of the dorsal dpp stripe requires independent inputs from the JNK pathway and from *pnr*.

Together, the preceding observations indicate that in late embryogenesis *pnr* acts as a positive regulator of both *ush* and *dpp*. These results also show that the regulatory interactions between *dpp* and *pnr* are reversed during development: whereas in early development *dpp* acts upstream *pnr* (Winick et al., 1993; Ashe et al., 2000), in late embryogenesis *pnr* upregulates *dpp* activity. This probably reflects the acquisition of different roles in the course of development.

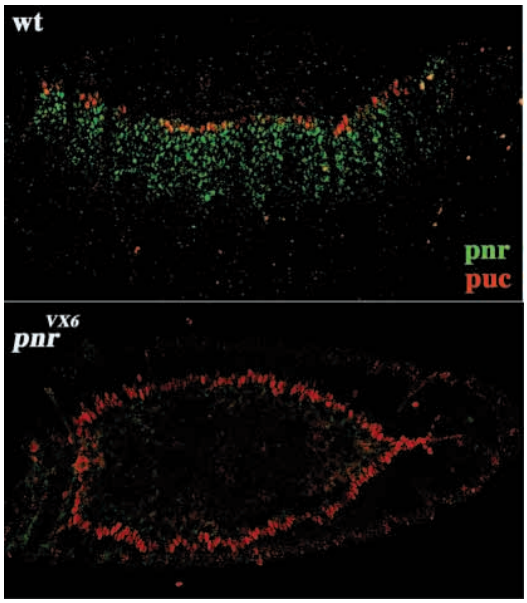

Fig. 6. Loss of activity of Pnr does not affect *puc* expression. The top picture is a lateral view of a wild-type late embryos doubly stained for *pnr* (green) and *puc* (red). The latter is expressed in a line of cells at the dorsal edge of the Pnr domain. The bottom picture is a dorsal view of a *pnr*^{VX6} mutant embryo showing normal Puc activity.

The Pnr product is ineffective in the amnioserosa

Despite its overall effect on the epidermis and the CNS, there is no detectable effect of *pnr* activity in the amnioserosa. For example, in *Ubx-Gal4/UAS-pnr* embryos the amnioserosa appeared to be unaffected even though it contains Pnr protein (Fig. 4C). Moreover, in those embryos there is an expansion of *dpp* expression all over the epidermis except in the amnioserosa (not shown), suggesting that *pnr* is unable to induce *dpp* activity there. We have explored this phenomenon by using a new Gal4 line, LP1, which drives high expression levels specifically in the amnioserosa (Fig. 7). In *LP1/UAS-pnr* embryos there is a high level of Pnr protein in the amnioserosa (Fig. 7B) but no sign of ectopic *dpp* expression (Fig. 7D). In addition, the expression of specific amnioserosa genes such as *Kr* is not affected (Fig. 7C) and germ band retraction is normal. This result suggests that the developmental function of *pnr* is inhibited in the amnioserosa at the post-transcriptional level. It resembles the phenomenon of phenotypic suppression/posterior prevalence, described for Hox gene function in the AP axis (Gonzalez-Reyes and Morata, 1990; Duboule, 1991; Duboule and Morata, 1994).

DISCUSSION

We have addressed the problem of how morphological diversity is achieved in the DV axis of the embryo. There are two pertinent questions to be answered: (1) how the embryo is subdivided into different parts along the DV axis; and (2) the identities of the genes responsible for making the various parts different from each other. Our results indicate that *pnr* is involved in the process: it participates in the subdivision of the dorsal region of the embryo into two distinct domains and also

specifies the identity of the dorsomedial domain. These results, together with those previously reported on *pnr* function in adult patterns (Calleja et al., 2000), strongly indicate *pnr* has a principal role in establishing the *Drosophila* body plan. We discuss these findings and also other aspects of the function and regulation of *pnr* during embryogenesis.

pnr expression and regulation during embryogenesis

In early development, *pnr* is activated in response to *dpp* activity (Winick et al., 1993; Ashe et al., 2000) in a broad dorsal domain, which we show extends from parasegments 2/3 to the border between 13/14, although the borders are not strictly parasegmental. The control by *dpp* is consistent with the effect of *brk* mutations on early *pnr* expression (Jazwinska et al., 1999b; Ashe et al., 2000).

The original expression domain is substantially modified during embryogenesis. By germ band extension (stage 10) *pnr* activity is limited dorsally by the border between the epidermis and the amnioserosa, and laterally by the dorsal border of *iro* (Calleja et al., 2000). We do not know which factor(s) is responsible for the loss of expression in the amnioserosa, although likely candidates are several genes specifically active in this region, such as *Race, zen, hindsight* or *serpent* (Frank and Rushlow, 1996; Rush and Levine, 1997). In addition, we do not know how the late expression is regulated at the lateral border. It is not achieved by *iro*, as the loss of the entire Iroquois complex does not affect *pnr* expression.

Another modification occurs between stages 10 and 11, and is the loss of expression in the A8 segment. Expectedly, it is under the control of *Abd-B*; in *Abd-B* mutants the gap in A8 does not appear (Fig. 2A). However, none of the known *Abd-B* target genes *sal*, *ems* and *grn* (Castelli-Gair, 1998) is involved in the regulation, as their mutations do not affect *pnr* expression. Our finding that *lin*, which is considered as a co-factor of *Abd-B* (Castelli-Gair, 1998), is involved (Fig. 2B), suggests that downregulation of *pnr* in the A8 segment is mediated either by an unknown *Abd-B* target or directly by interaction between the Abd-B and Lin products. It is not clear why *pnr* activity has to be eliminated precisely in the A8 segment. We notice that this segment gives rise to the spiracles, protruding structures that are very different from those differentiated by the other abdominal segments where *pnr* remains active. In fact, there are several *Abd-B* target genes specifically activated in the spiracles (Castelli-Gair, 1998). It is possible that the formation of these structures demands that the *pnr* activity, which specifies larval epidermis of very different morphology, be turned off.

Interestingly, whereas early *pnr* expression is under *dpp* control, the late expression is not. Late inactivation of the Dpp pathway, using a dominant negative form of *thick veins*, does not modify *pnr* expression. In addition, mutations at *brk*, which allow higher response levels to Dpp signalling (Campbell and Tomlinson, 1999; Jazwinska et al., 1999a; Minami et al., 1999) fail to affect *pnr* expression in late development (Marty et al., 2000; H. H. and G. M., unpublished), although they affect early expression (Jazwinska et al., 1999b; Ashe et al., 2000). This indicates that *pnr* expression is controlled independently in early and late development, and by different factors.

pnr functions during embryogenesis

There is already evidence that *pnr* has distinct functions during embryogenesis. Its activity in the dorsal epidermis is required for dorsal closure (Heitzler et al., 1996) and it is also expressed in the dorsal mesoderm where it is involved in the specification of cardiac cells (Gajewski et al., 1999).

We provide evidence for another and more general function of *pnr.* Our results indicate that it specifies the identity of a dorsomedial body region that spans from the labial segment to

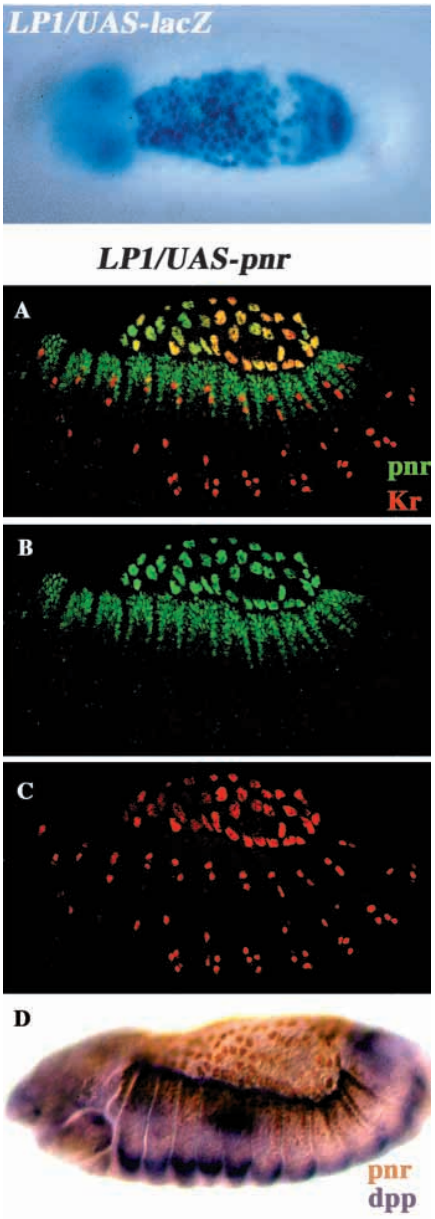

Fig. 7. Lack of effect on the Pnr protein in the amnioserosa. The LP1 line drives expression only in the amnioserosa, as indicated by the *LP1/UAS-lacZ* embryo shown on the top. (A) Confocal image of a doubly labelled embryo stained for *pnr* and *Kruppel*. (B,C) The green and red channels, indicate that although there are high levels of *pnr* in the amnioserosa (B) there is no effect on *Kr* expression (C). (D) Embryo of the same genotype doubly stained for *pnr* and *dpp*. The expansion of *pnr* expression to the amnioserosa does not modify *dpp* expression, which remains normal.

the end of the abdomen. This is clearly demonstrated by the effects seen in mutant embryos and after ectopic expression experiments. In pnr^{VX6} embryos, the dorsomedial cuticle does not form, and there is an expansion of the dorsolateral epidermis (Fig. 4), suggesting that the cells of the dorsomedial domain acquire a dorsolateral fate. The ectopic expression experiments also point to the same conclusion. In larvae like *arm-Gal4/UAS-pnr*, the entire larval epidermis acquires dorsomedial features (Fig. 4F,G), whereas using more restricted drivers (*Ubx-Gal4*, *wg-Gal4*) the transformation is limited to the region where the Pnr protein is present (Fig. 4D,E.H), suggesting that the effect of *pnr* is cell autonomous. Thus, the Pnr protein is able by itself to trigger a developmental pathway, a typical property of selector gene products (Mann and Morata, 2000). In addition, it induces a ventral to dorsal transformation as corresponding to each segment, indicating that it acts in combination with Hox genes. These observations indicate that selector genes in the AP and DV axes have to co-operate to determine the different spatial patterns.

The transformation of ventral and dorsolateral epidermis towards dorsomedial observed after ectopic *pnr* expression is also reflected in the activity of marker genes of the distinct regions. Characteristic genes of the ventral neuroectoderm such as BP102 for the CNS (not shown) or *buttonhead* (C. Estella and G. M., unpublished) are suppressed. In addition, *pnr* is able to suppress *iro* activity (Calleja et al., 2000), a property that, as in the adult cells, is important to keep the dorsomedial and dorsolateral domains separate during embryogenesis.

The developmental effects observed after either loss or the gain of *pnr* function in the larval epidermis resemble those reported for the adult cuticle. In the latter, it has been shown that the activity of *pnr* maintains the segregation of the dorsal cuticle into medial and lateral domains, and also specifies the identity of a medial one (Calleja et al., 2000). This indicates that *pnr* has a general function involved in the subdivision of the body along the DV axis. The longitudinal stripe of *pnr* expression established during embryogenesis is probably a major constituent of the body and represents an zone of common identity.

In addition, Pnr has other more concrete functions connected with the specification of cardiac cells (Gajewski et al., 1999) and embryonic dorsal closure (Heitzler et al., 1996). Our results indicate that the involvement of *pnr* in dorsal closure is exerted through its activation of *dpp* in late embryogenesis, which is responsible for the formation of the Dpp stripe at the junction of the epidermis with the amnioserosa. Normal functioning of the Dpp pathway in this region is required for dorsal closure (Affolter et al., 1994; Glise and Noselli, 1997), suggesting that defects in dorsal closure observed in *pnr* mutant embryos (Heitzler et al., 1996) is the result of the lack of the dorsal dpp stripe.

There is evidence that this *dpp* expression requires function of the JNK kinase pathway (Glise and Noselli, 1997), and we show that it also requires *pnr* activity. Our observation that in absence of *pnr* activity the expression of *puc*, the end element of the JNK pathway (Martin-Blanco et al., 1998) is normal, indicates that in *pnr* mutants the JNK pathway is normally active. In turn, it shows that the activation of *dpp* in the dorsal stripe requires independent inputs from both the JNK pathway and *pnr*.

Phenotypic suppression of *pnr* in the amnioserosa?

One intriguing aspect of *pnr* function is that it is able to induce a developmental modification in all ectodermal structures along the DV body axis except in the amnioserosa, the most dorsal tissue. Even under conditions in which *pnr* is transcribed and translated in all the amnioserosa cells (Fig. 7), it does not appear to elicit any developmental effect; none of the amnioserosa marker genes is affected by forcing *pnr* activity and the retraction of the germ band [a morphological indicator of the function of specific amnioserosa genes (Frank and Rushlow, 1996)] is also normal. Similarly, *pnr* is able to induce *dpp* activity all over the body except in the amnioserosa (Fig. 5, Fig. 7), where the presence of the Pnr protein appears to be inconsequential. This situation resembles the phenotypic suppression/posterior prevalence phenomenon discovered in the Hox genes specifying the AP body axis (Gonzalez-Reyes and Morata, 1990; Duboule, 1991; Duboule and Morata, 1994). It consists of a functional inactivation of a Hox protein by the presence of another normally expressed in a more posterior region of the body. It is conceivable that there might be a 'dorsal prevalence' in the DV axis, by which dorsal expressing genes are functionally dominant over the ventral expressing ones. It would be expected that genes specifying amnioserosa would be able to transform all structures as they would be ranking highest in the functional hierarchy.

We thank Juan Pablo Albar for his help with the analysis of the amino acid sequence of Pnr, for advise on preparing the anti-Pnr antibody and the generous gift of the peptides. We thank Mariann Bienz for the *UAS-pnr* flies. We also thank Natalia Azpiazu, Manuel Calleja, Carlos Estella and Ernesto Sanchez-Herrero for discussions and comments on the work, and Rosa Gonzalez and Angélica Cantarero for their technical help. The experimental work has been supported by grants from the Dirección General de Investigación Científica y Técnica, the Comunidad Autónoma de Madrid and an institutional grant from the Fundación Ramón Areces.

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