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THE SPECIFICATION OF DORSAL AND LATERAL PATTERN  
IN THE *DROSOPHILA* EMBRYO

A THESIS PRESENTED

BY

CAMILA VICENCIO ESGUERRA

TO

THE OPEN UNIVERSITY  
MILTON KEYNES, ENGLAND

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## ABSTRACT

The dorsal-ventral axis of the *Drosophila* embryo is established through the nuclear gradient expression of the maternal morphogen *dorsal*, which sets up the dorsal, lateral, and ventral domains along the embryo circumference. The steps from this maternal signal to the onset of patterning and morphogenesis is quite rapid, and although genetic approaches have identified several zygotic genes that are transcriptionally regulated by *dorsal*, the direct targets of these zygotic genes, as well as other targets of *dorsal*, are still unknown.

A molecular screen was utilized in order to identify genes that are differentially expressed along the dorsal-ventral axis, with a specific focus on dorsal-lateral genes involved in patterning and differentiation. A subtractive cDNA library highly enriched for genes expressed in the dorsal and lateral regions of the embryo was screened twice. From the two screens, 24 non-crosshybridizing clones were obtained, 21 of which appear to be novel genes based on preliminary sequence analysis and *in situ* hybridization patterns. Two clones turned out to be cDNA fragments of previously identified genes. One clone had an identical sequence to the *Drosophila* POU domain genes *pdm-1* and *pdm-2*, while the other turned out to be *tolloid*, one of the dorsal-ventral patterning genes.

In conjunction with previously described genes, several clones from the library were then chosen to serve as molecular markers for specific domains along the dorsal-ventral axis. *In situ* hybridizations were performed on a series of mutant embryos either lacking progressively larger portions of the dorsal part of the embryo (zygotic ventralizing mutants), or lacking the entire mesoderm (*twist snail* double mutants). The aim of this thesis was to understand how patterning along the dorsal-ventral axis occurs, and to determine whether the current model on how the genes for the zygotic ventralizing mutants interact with one another to pattern the dorsal part of the embryo is correct. Indeed, a number of results show that this model may have to be revised.

"Beware of determining and declaring your opinion suddenly on any object; for imagination often gets the start of judgement, and makes people believe they see things, which better observations will convince them could not possibly be seen: therefore assert nothing till after repeated experiments and examinations in all lights and in all positions. When you employ the microscope, shake off all prejudice, no harbour any favourite opinions; for, if you do, 'tis not unlikely fancy will betray you into error, and make you think you see what you would wish to see. Remember that truth alone is the matter you are in search after; and if you have been mistaken, let not vanity seduce you to persist in your mistake."

Baker, H. (1742). A supplement to the account of a distempered skind, published in the 424th number of the *Philosophical Transactions*. *Philosophical Transactions* 49, 21-24

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CHAPTER 1  
INTRODUCTION



## INTRODUCTION

Positional information along the dorsal-ventral axis of the *Drosophila* embryo is established through the coordinated action of a set of thirteen maternal genes: *nudel* (*nud*), *pipe* (*pip*), *windbeutel* (*wind*), *easter* (*ea*), *gastrulation defective* (*gd*), *snake* (*sna*), *spätzle* (*spz*), *Toll* (*Tl*), *tube* (*tub*), *pelle* (*pel*), *cactus* (*cact*), and *dorsal* (*dl*) (reviewed in Anderson, 1987). The first twelve maternal genes act along a single cascade to establish a nuclear activity gradient of the thirteenth gene, the *dl* gene (Steward et al., 1988). This is accomplished through the differential activation of the *dl* protein along the dorsal-ventral axis. In the syncytial blastoderm embryo, the highest levels of active *dorsal* protein are present in ventral nuclei while in dorsal nuclei, the *dl* protein is excluded and remains inactive in the cytoplasm (Roth et al., 1989; Steward, 1989; Rushlow et al., 1989).

The *dl* protein shares structural homology to the mammalian transcription factor NF- $\kappa$ B and the avian *rel* oncogene, (Steward, 1987; Ghosh et al., 1990; Kieran et al., 1990) and acts as a transcriptional regulator by binding to *cis*-regulatory sequences of certain downstream zygotic genes (Ip et al., 1991; Thisse et al., 1991). The presence of high levels of *dl* protein in ventral nuclei activate the zygotic genes *twist* (*twi*) and *snail* (*sna*), while its absence in dorsal nuclei allows for the expression of the zygotic genes *decapentaplegic* (*dpp*) and *zerknüllt* (*zen*) (Thisse et al., 1987; Rushlow et al., 1987; Roth et al., 1989; Steward, 1989; Ray et al., 1991). Thus, the *dl* nuclear activity gradient defines a primary pattern of gene expression. Dorsal cells express *dpp* and *zen* but not *twi* and *sna*, lateral cells express none of the four genes, and ventral cells express *twi* and *sna*, but not *dpp* and *zen*.

In the cellular blastoderm embryo, *twi* and *sna* are expressed in a continuous longitudinal stripe that occupies the ventral 20% of the embryo. The cells within this region comprise the cells of the mesoderm primordium. *twi* transcripts can also be detected outside the presumptive mesoderm and the overall pattern appears more as a steep gradient with the highest levels of expression within the mesoderm primordium. *sna* transcripts on the other hand are strictly contained within the presumptive mesoderm. This is achieved through the action of both *twi* and *dl* which specify the sharp border of *sna* expression (Ip et al., 1992). *twi* encodes a helix-loop-helix protein while *sna* encodes a zinc finger protein, and both zygotic genes function in a complementary manner to specify mesodermal fates (Nüsslein-Volhard, 1984; Thisse et al., 1988; Alberga et al., 1991). *twi* activates other downstream zygotic genes required for mesoderm differentiation while *sna* blocks other non-mesodermal genes from being expressed in the mesoderm (Kosman et al., 1991; Leptin, 1991).

Two examples of such genes repressed by *sna* are *single-minded (sim)* and *rhomboid (rho)* (Nambu, et al., 1990; Nambu, et al., 1991; Bier et al., 1990). The *sim* gene encodes a helix-loop-helix protein and plays a key role in the differentiation of the mesectoderm, while *rho* encodes a putative transmembrane protein and is also involved in the differentiation of the mesectoderm as well as a subset of ventral epidermal cells within the neurogenic region. Like *twi* and *sna*, both genes have also been shown to be direct targets of *dl* (Ip et al., 1992). Thus, the *dl* nuclear activity gradient functions as a morphogen to promote specific cell fates along the ventral side of the embryo by differentially activating zygotic patterning genes. High levels of *dl* activity promote mesodermal fates, while intermediate levels promote mesectodermal and neuroectodermal fates. More recently, it has been shown that a narrower region within the *twi* expressing domain exists. This ventral domain is where the *folded gastrulation* gene (*fog*) is expressed and is also believed to be directly controlled by *dl*. Hence, the *fog* domain may define yet another region in which the *dl* activity gradient is interpreted.

As mentioned above, the absence of *dl* protein in dorsal nuclei allows for the expression of dorsally expressed zygotic genes such as *dpp* and *zen*. In the syncytial blastoderm embryo, both genes are expressed along the dorsal 40% of the embryonic circumference. However, by the onset of cellularization *zen* expression refines to the region that will later become the amnioserosa, and thus the dorsal side is subdivided into presumptive amnioserosa and dorsal ectoderm. Since no maternal morphogen is present on the dorsal side, then the question arises as to how this subdivision along the dorsal side is created. The expression of genes on the ventral side is directly regulated by the *dl* nuclear gradient, but this cannot be the case on the dorsal side since *dl* is absent from the nucleus and zygotic genes are initially expressed in a broad domain along the dorsal side. Therefore a secondary level of patterning must occur on the dorsal side and must occur through the action of zygotic genes.

### ***dpp* gradient model**

Based on their mutant phenotypes, seven zygotic loci have been identified that are postulated to play a major role in the specification of dorsal pattern elements: *dpp*, *tolloid (tld)*, *screw (scw)*, *shrew (srw)*, *short gastrulation (sog)*, *twisted gastrulation (tsg)*, and *zen*. (reviewed by Rushlow and Arora, 1990). Loss-of-function alleles of any one of these genes results in the loss of dorsal pattern elements to varying degrees and the subsequent expansion of more ventral pattern elements. Thus, these mutants have been classified under the category of zygotic ventralizing mutants. Loss-of-function alleles of *dpp* result in the most severe phenotype with the loss of all dorsal



pattern elements, while loss-of-function alleles of *tld* and *scw* result in the loss of the amnioserosa and portions of the dorsal epidermis. Loss-of-function alleles of *srw*, *sog*, *tsg*, and *zen*, result in the loss of the amnioserosa.

Of these seven zygotic genes, *dpp* is considered to be unique. Not only do null alleles of *dpp* result in the most severe mutant phenotype, but the expression of *dpp* is also independent of any of the other zygotic genes. However, the refined expression of at least *tld* and *zen* is dependent on *dpp* activity (Ray et al., 1991; Ray, 1993). Furthermore, analysis of genetic interactions between *dpp* and the other zygotic genes revealed that *zen* acts downstream of *dpp*, while *tld* and *srw* function to enhance *dpp* activity, and *sog* functions to repress *dpp* activity (Ferguson and Anderson, 1992). Thus, given these data, it has been proposed that these seven genes act along a single patterning pathway with *dpp* as the central element. Genetic and molecular analysis of *dpp* function revealed that patterning along the dorsal-ventral axis is highly sensitive to the amount of *dpp* activity. Amorphic alleles of the *dpp* gene result in the severe loss of dorsal pattern elements and a significant expansion of ventral pattern elements, while hypomorphic alleles of *dpp* result in a weaker phenotype with only a slight loss in dorsal pattern elements (Wharton et al., 1993). More recent experiments have shown that increasing the activity of *dpp* through RNA injections in lateralized embryos results in the expansion of the amnioserosa. These data suggest that *dpp* acts in a graded fashion where high levels specify amnioserosa, intermediate levels specify dorsal epidermis, and lower levels specify dorsal neuroectoderm.

Thus, *dpp* is likened to the maternal gene *dl* in its function as a morphogen for the dorsal side of the early embryo. The model also suggests that any lowering of the *dpp* gradient results in the loss of dorsal pattern elements and a corresponding expansion of more ventral pattern elements to a more dorsal position. In other words, the model postulates that the mutant phenotypes of the zygotic ventralizing mutants can be described as simple fate shifts along the dorsal-ventral axis due to the decrease in *dpp* activity in these mutants. However, in order to determine whether only simple fate shifts occur, one has to look at earlier markers rather than cuticular patterns to follow the boundaries that mark the subdivisions along the dorsal-ventral axis. One way to follow early boundaries is to look at morphological boundaries based on early cell division patterns and cell shape changes. In addition, gene expression boundaries can be determined through the analysis of the expression patterns of the zygotic patterning genes, as well as their downstream targets.

## DIFFERENTIATION OF REGIONS ALONG THE D-V AXIS USING A VARIETY OF EARLY MARKERS

### Cell shape changes

Until just prior to the end of cellularization all cells along the dorsal-ventral axis within the trunk region of the embryo appear morphologically identical, except for the fact that ventral cells cellularize faster than dorsal cells. Immediately after cells on the ventral side have cellularized, gastrulation begins and the first morphological differences become apparent. The cells which occupy the ventral-most 20% of the embryo constrict their apices leading to a distinct flattening of the ventral side. Simultaneously, nuclei within these cells are shifted basally and the cells elongate to form the ventral furrow. Concomitantly, contraction of these cells occurs as well as a buckling of the epithelium and the invagination of cells into the embryo interior. These cells comprise the mesoderm and eventually form a uniform cell layer just beneath the ectoderm (Leptin and Grunewald, 1990; Sweeton, 1991).

By the time the mesodermal cells have formed a tube inside the embryo (late stage 6), the ectodermal cells have flattened slightly and appear cuboidal. Between stage 8 and early stage 9 when the mesodermal cells have dispersed and have begun to divide, three regions of cells can be distinguished within the ectoderm. The region of cells that flanked the mesoderm on each side have been brought to lie next to each other along the ventral midline due to the invagination of the mesoderm. These cells total approximately 18 cells in width (early cross-sections, Hartenstein and Campos-Ortega, 1985 and this work) resume their tall and columnar appearance and appear significantly larger than all the other cells along the embryonic circumference. This region corresponds to the ventral neuroectoderm and can be further subdivided into three clusters according to the manner in which the three waves of neuroblasts arise in later stages: *NRl*, *NRm*, and *NRi* (Hartenstein and Campos-Ortega, 1985). Around the same time, the cells just dorsal to the neuroectoderm undergo their first mitotic division. These cells appear small and round and correspond to the dorsal epidermis. The most dorsal cells correspond to the amnioserosa and appear very flat and thin.

By fully extended germ band an additional cell population is morphologically distinguishable, the mesectodermal cells. These cells originate as two rows of cells bordering the mesodermal primordium in the cellular blastoderm and are brought together at the ventral midline after the mesoderm has invaginated. By fully extended germ band, they constrict at their apices and appear tear-drop shaped. Thus, based on these morphological criteria, the dorsal-ventral axis can be subdivided into five distinct

regions, the mesoderm, the mesectoderm, the ventral ectoderm, the dorsal ectoderm, and the amnioserosa.

### **Mitotic domains**

The first 13 cleavage divisions within the fertilized embryo are synchronous. As soon as cellularization is completed this synchrony ceases and different groups of cells enter the 14th mitotic cycle at various times in a specific spatial and temporal order. These localized regions that undergo synchronous mitosis are called the mitotic domains (Hartenstein and Campos-Ortega, 1985; Foe, 1989). This complex pattern of synchronized mitoses occupies regions along the two main body axes, exhibits bilateral symmetry, and is highly reproducible. The cells within a specific domain share common attributes such as cell morphology, morphogenetic behavior, and differentiated cell fates (Foe, 1989). Thus, mitotic domains can be used as markers for differentiated cell fates within the early embryo.

More than 25 different mitotic domains have been identified for the 14th mitotic cycle, and each domain is numbered based on the time it enters the M phase of this mitotic cycle. Hence, the first set of cells to enter mitosis 14 is  $\partial_{141}$  and the second,  $\partial_{142}$ , and so forth. Most of the 25 mitotic domains lie within the acron, procephalon and telson, but only the seven mitotic domains within the segmented region (dorsal-ventral axis) will be considered here.

The first of these seven domains to divide is  $\partial_{1410}$ . This is the largest of all the domains and comprises all cells within the mesoderm promordium. The cells within this domain divide shortly after they have internalized with the ventral furrow. Just after the cells of the mesoderm have entered mitosis 14, mitosis begins simultaneously at 5 dorsal-lateral sites along each side of the embryo. Mitotic waves within these sites spread out in an anterior-posterior direction, resulting in a continuous longitudinal stripe directly behind the cephalic furrow. This domain is called  $\partial_{1411}$  and includes cells that will form the dorsal epidermis and the tracheal tree. Thus, during the time that cells within  $\partial_{1411}$  are dividing, the embryo can be divided into four distinct regions. The cells of the mesoderm have already invaginated and are thus separate from the ectoderm. The cells of  $\partial_{1411}$  appear small and cuboidal and lie between the flattened cells of amnioserosa and the enlarged ventral ectodermal cells. Hence, there is a direct correlation between cell shape changes and mitotic division patterns. In later stages however, this pattern is further refined by the appearance of two other mitotic domains. The first domain,  $\partial_{1414}$  corresponds to the cells that will form the mesectoderm and are the cells that are brought together after the invagination of the presumptive mesodermal cells (see above). The second domain,  $\partial_{1419}$ , enters mitosis



at the end of rapid germ band extension and consists of a single row of cells on each side of the embryo that lies just dorsal to  $\partial_{14}11$ . This domain has been termed the dorsal edge since it marks the boundary between dorsal epidermis and amnioserosa (Ray, 1993). The amnioserosa has been designated  $\partial A$ , although strictly speaking, it is not a true mitotic domain since none of the cells within this region ever divide. However, the cells within this region are morphologically distinct from cells in other regions, and the boundary between amnioserosa and the dorsal epidermis is defined by the entrance of  $\partial_{14}19$  cells into mitosis.

The last set of cells to divide are the cells of the neurogenic ectoderm. This region, although composed of smaller segmentally repeated domains can be subdivided into two major domains,  $\partial_{14}N$  and  $\partial_{14}M$ . The cells within  $\partial_{14}N$  encompass the dorsal half of the neurogenic ectoderm and enter mitosis during early stage 9. This domain lies just ventral to  $\partial_{14}11$ . Cells within  $\partial_{14}M$  enter mitosis at the start of stage 10 and lie between  $\partial_{14}N$  and  $\partial_{14}10$ .

Thus, proceeding from ventral to dorsal, the early embryo can be subdivided into seven domains based on mitotic pattern:  $\partial_{14}10$ ,  $\partial_{14}14$ ,  $\partial_{14}M$ ,  $\partial_{14}N$ ,  $\partial_{14}11$ ,  $\partial_{14}19$ , and  $\partial A$ . These domains correspond to the mesoderm, mesectoderm, ventral neuroectoderm, dorsal neuroectoderm, dorsal epidermis, dorsal edge, and amnioserosa respectively. Similarly, boundaries between these domains can also be defined. The  $\partial A/\partial_{14}11$  boundary corresponds to the amnioserosa/dorsal epidermis boundary (dorsal edge) and aligns with  $\partial_{14}19$ , the  $\partial_{14}11/\partial_{14}N$  boundary corresponds to the dorsal epidermis/neuroectoderm boundary, and the  $\partial_{14}M/\partial_{14}10$  boundary corresponds to the neuroectoderm/mesoderm boundary (mesectoderm), and aligns with  $\partial_{14}14$ .

### **Expression patterns of select zygotic genes**

It is not surprising that groups of cells that have embarked along common developmental pathways not only share identical cell division patterns and undergo the same morphological cell shape changes, but also express the same genes. Using the above mentioned genes as markers for cell fate, we can fill in the various domains along the dorsal-ventral axis. On the ventral side, mesodermal cells express *twi* and *sna*, mesectodermal cells express *sim*, and ventral neuroectodermal cells express *rho*. On the dorsal side, *dpp* transcripts are initially detected over the dorsal 40% of the embryo circumference, but by mid-stage 8, they are excluded from the amnioserosa, and can therefore be used as a marker for dorsal epidermis. Early *zen* transcripts are also present over the same broad domain as early *dpp* transcripts. However, before the onset of cellularization, *zen* expression is confined to the amnioserosa, making it a

good early marker for this region (Ray et al., 1991; St. Johnston and Gelbart, 1987; Rushlow et al., 1987). Thus, the refined expression of *dpp* and *zen* mark the dorsal epidermis and amnioserosa fates respectively. Notably, it is still not known what specifies the region between *rho* and *dpp*.

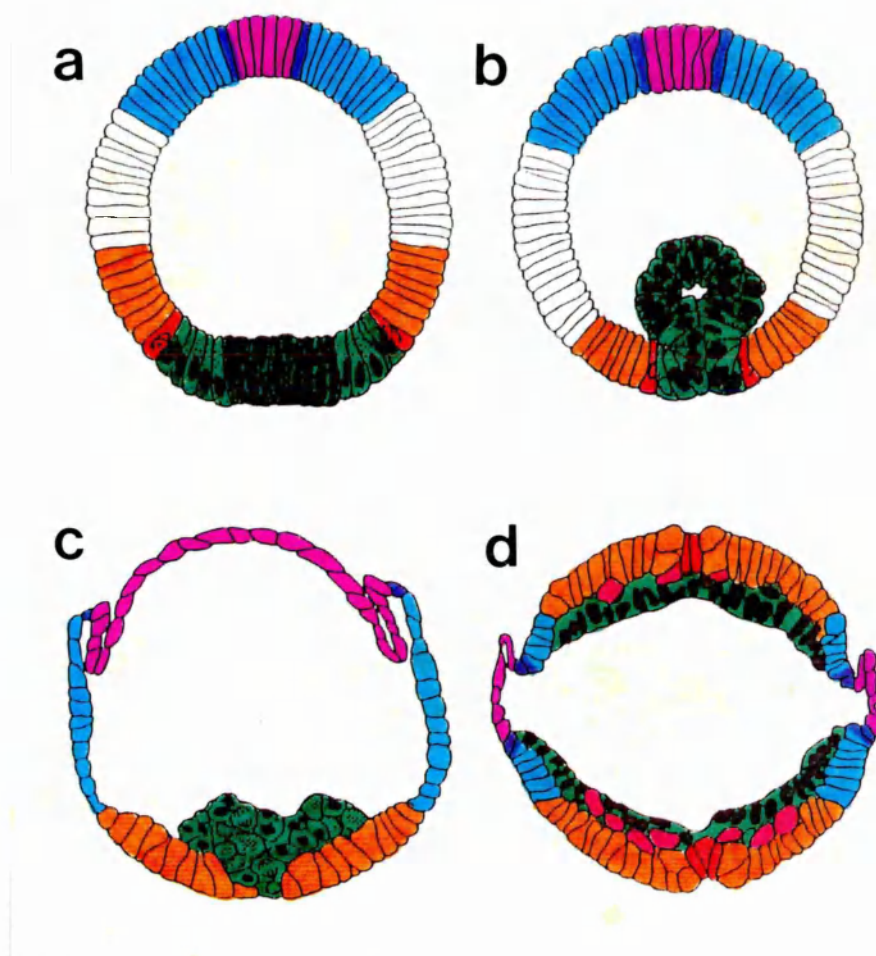
## AIM OF THIS THESIS

As mentioned previously, the *dpp* gradient model was formulated mainly on the basis of cuticular phenotypes which have been projected onto the blastoderm embryo. However, the analysis of cuticular patterns is not sufficient to fully characterize or distinguish between the similar phenotypes displayed by the zygotic ventralizing mutants. The occurrence of massive cell death, fate shifts, transformations, and incomplete gastrulation movements makes it very difficult to determine which cells from the blastoderm embryo correlate to the cells that appear within the same region in older embryos and in the larva. Thus, in order to identify fate shifts among groups of cells in these mutants one has to use other markers at earlier stages to test the existing model. Furthermore, it is not clear exactly how these zygotic ventralizing genes function and whether they have other functions aside from dorsal-ventral patterning.

Experiments by Arora and Nüsslein-Volhard used the cell division pattern of cells during the 14th mitotic cycle as early cell fate markers. The mitotic patterns were analyzed in the context of zygotic ventralizing mutants and were found to correlate with the cell fate changes observed in the later mutant cuticular phenotypes (Arora and Nüsslein-Volhard, 1992). However, discrepancies between these results and results from experiments done by others still exist. Work by Arora & Nüsslein-Volhard described *srw* mutants as missing the entire amnioserosa region, whereas work by Ray (1993) described *srw* mutants as missing only parts of the amnioserosa, as evidenced by partial staining of *Krüppel*, a gene expressed in amnioserosa cells. Furthermore, work by Rao et al. (1991) described a ventral shift of a series of neuronal markers in *twi sna* double mutants, whereas work by Arora and Nüsslein-Volhard described no such shift.

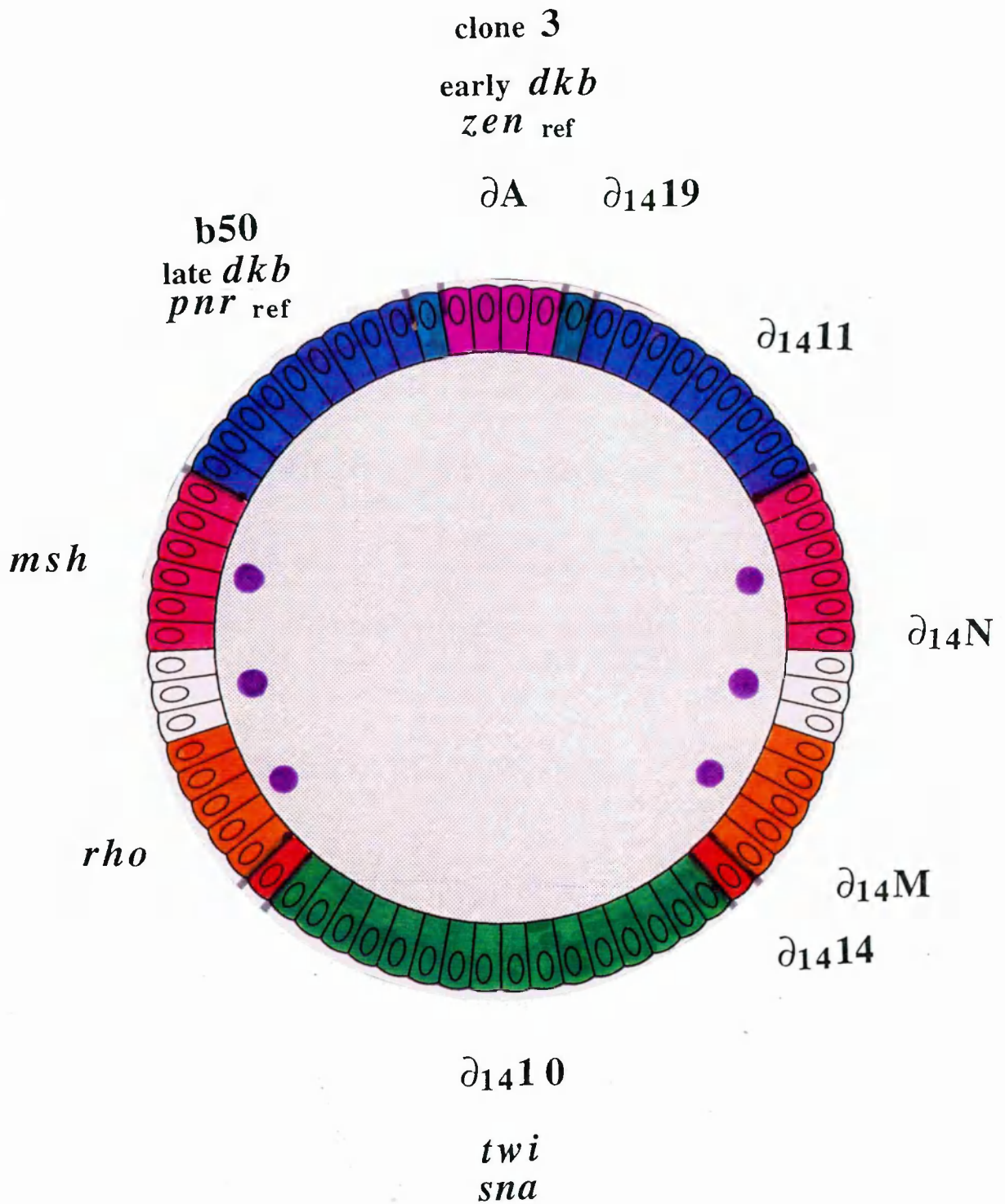
In light of these discrepancies we decided to reinvestigate what occurs in these zygotic patterning mutants. We therefore decided to use select clones obtained from a subtractive cDNA library highly enriched for dorsal and laterally expressed genes in conjunction with other previously identified genes as molecular markers for early cell fate. In addition we were curious to see how these dorsal-lateral specific genes fit into the patterning cascade. Morphological criteria were first used to determine whether these markers were truly within specific domains. Then, the expression patterns of these early markers were analyzed in the context of the zygotic ventralizing mutants

and *twi sna* double mutants. From the data obtained, we found that some results did not correlate with the existing model. Some data suggests that in certain cases there is not a simple expansion of ventral pattern elements to a more dorsal position and that patterning on the dorsal side does not occur through one pathway alone, but rather through parallel pathways.



**Figure 1.1. Early cell shape changes and mitotic domains as morphological markers along the dorsal-ventral axis.** Camera lucida drawings of embryonic cross-sections at four different stages of development. The amnioserosa is shown in pink ●. This is also the region where *zen* expression refines and is designated  $\partial A$  even though it is not truly a mitotic domain. The dorsal epidermis is depicted in dark blue and blue ●●, and corresponds to  $\partial_{1419}$  and  $\partial_{1411}$  respectively. This is also the region where *dpp* expression refines. The ventral neuroectoderm ( $\partial_{14N}$ ) is shown in orange ●. *rho* expression covers approximately the ventral third of this region as well as the mesectoderm. The mesectoderm ( $\partial_{1414}$ ) is shown in red-orange ●, and is where *sim* is expressed. The mesoderm ( $\partial_{1410}$ ) shown in green ●, is where *twi* and *sna* are expressed. (A) Stage 5 embryo. Cellularization has just been completed and the cells of the mesoderm constrict their apices leading to a distinct flattening of the ventral side. (B) Late stage 6 embryo. The mesodermal cells have formed a tube and the ectodermal cells have flattened slightly and appear cuboidal. (C) Stage 8 embryo. The mesodermal cells have divided ( $\partial_{1410}$ ) and are beginning to disperse. The cells of the dorsal epidermis round up and begin to divide ( $\partial_{1411}$ ). Shortly after, the cells of the mesectoderm divide ( $\partial_{1414}$ ). The cells of the neurogenic region enlarge, while the cells of the amnioserosa become very flat and thin. At the end of stage 8, a single row of cells in the most dorsal epidermis divide ( $\partial_{1419}$ ) as well as the cells of the neurogenic region ( $\partial_{14N}$ ). (D) Stage 10 embryo. The cells of the mesectoderm have been brought together due to the invagination of the mesoderm and appear tear-drop shaped. The cells of the mesoderm have spread out and the neuroblasts have begun to delaminate. ● The cells in the ventral third of the neurogenic region divide ( $\partial_{14M}$ ).





**Figure 1.2. Expression patterns of select zygotic genes as markers for early cell fate along the dorsal-ventral axis.** Gene expression patterns are projected onto a cross-section of the blastoderm embryo. From dorsal to ventral: early *dkb* (pink)● as a marker for the amnioserosa ( $\partial A$ ), late *pnr* (dark blue and blue)● as a marker for the dorsal epidermis ( $\partial_{1419}$  and  $\partial_{1411}$ ), *msh* (red)● as marker for the dorsal third of the neuroectoderm ( $\partial_{14N}$ ), *rho* (orange, red-orange)● as a marker for the ventral third of the neuroectoderm and the mesectoderm ( $\partial_{14M}$ ,  $\partial_{1414}$ ), and late *sna* (purple)● as a marker for neuroblasts. The late *dkb* expression as well as the dorsal-lateral clone b50 were also used as markers for the dorsal epidermis. Clone 3 was used as a marker for the amnioserosa proper. The mesoderm is colored in green.●

CHAPTER 2  
MATERIALS AND METHODS



## MATERIALS AND METHODS

### 2.1. Materials

#### Chemicals

Acrylamide	Sigma, St. Louis, USA
Agar	Gibco BRL, Eggenstein
Agarose	Gibco BRL, Eggenstein
Ampicillin	Sigma, St. Louis, USA
Bacto yeast extract	Difco
Bacto Tryptone	Difco
Bromphenolblue	Sigma, St. Louis, USA
Digoxigenin-11-dUTP	Boehringer Mannheim
N,N'-Dimethylformamide	Sigma, St. Louis, USA
dNTPs	Boehringer Mannheim
Ethidium bromide	Sigma, St. Louis, USA
Glycine	Sigma, St. Louis, USA
Isopropanol	Roth, Karlsruhe
Klorix	Dan Chemie, Hamburg
2-Mercaptoethanol	Sigma, St. Louis, USA
NBT	Sigma, St. Louis, USA
Orange G	BDH Chemicals
phenol	Roth, Karlsruhe
Sodium dodecyl sulfate (SDS)	Serva, Heidelberg
N,N,N,N-Tetramethylenediamine (TEMED)	Sigma, St. Louis, USA
Tris	Biomol
Triton X-100	Serva, Heidelberg
tRNA	Boehringer Mannheim
Tween 20	Sigma, St. Louis, USA
Voltalev oil PCTFE, 10 S	Atochem, Pierre-Benite, France
X-phosphate	Boehringer Mannheim

All organic solvents were purchased from Merck, Darmstadt.

#### Radiochemicals

$\alpha$ [ <sup>35</sup> S] dATP	Amersham
$\alpha$ [ <sup>32</sup> P] dCTP	Amersham

#### Enzymes

Klenow fragment, <i>E. coli</i> (2U/ $\mu$ l)	Boehringer Mannheim
Eco RI (10U/ $\mu$ l)	Boehringer Mannheim
T4 DNA Ligase	Boehringer Mannheim
Calf intestinal alkaline phosphatase	Boehringer Mannheim
Proteinase K	Sigma, St. Louis, USA

#### Kits

Sequenase 2.0 kit	United States Biochemicals
T7 Quick Prime kit	Amersham
Qiagen plasmid kit	Qiagen, Düsseldorf

Films

Agfapan APX 100	Agfa-Gevaert, Leverkusen
Kodak Ektachrome 320 or 160T	Eastman Kodak, NY, USA
Kodak film cassettes with intensifying screen	Eastman Kodak, NY, USA
Polaroid type 667, ISO 3000	Polaroid, Cambridge, MA, USA
Röntgenfilm X-Omat AR	Eastman Kodak, NY, USA
Röntgen film developer	Tetenal, Norderstedt
Röntgen film fix	Tetenal, Norderstedt

Equipment

Axiophot microscope	Zeiss
microtome 2050 SUPERCUT	Reichert-Jung
centrifuge	Hereaus

**2.1.1. Buffers and solutions**

acrylamide solution	30% acrylamide stock (acrylamide:Bisacrylamide 38:1)
Aqua "bidest"(ddH <sub>2</sub> O)	tap water, double-distilled with Millipore ion exchanger
DNA loading buffer	0.25% Orange G, 0.25% Xylene Cyanol FF, 15% Ficoll type 400 in ddH <sub>2</sub> O.
hybridization mix	50% deionized formamide, 5X SSC, 0.5% tRNA (20mg/ml), 1% salmon sperm DNA (10mg/ml), 0.1% Tween 20 in ddH <sub>2</sub> O.
staining buffer	100mM NaCl, 50mM MgCl <sub>2</sub> , 100mM Tris-HCl pH 9.5, 0.1% Tween 20 in ddH <sub>2</sub> O.
LB Medium	10g Bacto-Tryptone, 5g Bacto yeast extract, 10g NaCl, with NaOH pH to 7.0, and autoclave in 1liter ddH <sub>2</sub> O.
NBT solution	10mg/ml Nitroblue tetrazolium, 70% dimethylformamide
X-Phosphate	10mg/ml 5-Bromo-4-Chloro-Indoylphosphate in dimethylformamide
PBS	70mM Na <sub>2</sub> HPO <sub>4</sub> , 30mM NaH <sub>2</sub> PO <sub>4</sub> , 130mM NaCl, pH7.1
PBT	0.1% Tween 20 in PBS
Phenol/Chloroform	Phenol/Chloroform/Isoamylalcohol 25:25:1, saturated TE
20X SSC	3M NaCl, 330mM Na-Citrate, pH to 7.0 with NaOH
TBE	45mM Tris/Borate, 1mM EDTA
TE	10mM Tris-HCl pH 7.5, 1mM EDTA

## 2.1.2. Fly Stocks and cDNAs

*white* flies were used as wild-type (Hazelrigg, 1987). Unless otherwise noted, all mutants used were obtained from the Tübingen stock collection. The following mutant alleles were used: *sog*<sup>XM42</sup>, *tsg*<sup>YB</sup>, *tld*<sup>10E95</sup>, *srw*<sup>10K28</sup>, *scw*<sup>C13</sup>, *zen*<sup>W36</sup>, *sna*<sup>IIIG</sup> Df(2R)*twi*<sup>S60</sup> (*twist snail* double mutant, provided by Kavita Arora), Df(2L)DTD2/Dp(2;2)MVD2 (*dpp*), and Dp(2,1)G146/+;*dpp*<sup>37</sup>. *dpp* mutants were created either by crossing the Df(2L)DTD2/Dp(2;2)MVD2 stock to itself to obtain a homozygous deficiency covering the *dpp* region, or by crossing it to the Dp(2;1)G146/+;*dpp*<sup>37</sup> stock (see below). Fly stocks were grown and collected under standard conditions (Ashburner, 1989; Wieschaus and Nüsslein-Volhard, 1986). The *pnr* cDNA (2.95 kb HindIII-EcoRI fragment in pBluescript SK+) was provided by Pat Simpson. The *snail* cDNA clone in pNB40 (Brown and Kafatos, 1988) was provided by Nick Brown. The *string* probe (Digoxigenin labeled BamHI 1-1650bp cDNA fragment) was provided by Christian Lehner. The *msh-1* cDNA was provided by Manfred Frasch.

## 2.2. Methods

### 2.2.1. *dpp* mutant cross

**D713** Dp (2;1) G146/+; *dpp*<sup>37</sup> Sp cn bw/CyO

**D707** Df (2L) DTD2/Dp (2;2) MVDL, ast ed dp cl

P1 ♀ +/+; Df/Dp (2;2) x Dp (2;1)/Y; *dpp*<sup>37</sup>/CyO ♂

F1 ♀ Dp(2;1)/+ : Df/*dpp*<sup>37</sup> Df/CyO Dp(2;2)/*dpp*<sup>37</sup> Dp(2;2)/CyO  
 ♂ +/Y : Df/*dpp*<sup>37</sup> Df/CyO Dp (2;2)/*dpp*<sup>37</sup> Dp(2;2)/CyO

♀ +/+; Df/Dp (2;2) x +/Y; Dp (2;2)/*dpp*<sup>37</sup> ♂

F2 Df/Dp (2;2) Dp (2;2)/Dp (2;2) Dp (2;2)/*dpp*<sup>37</sup> Df/*dpp*<sup>37</sup>

### **2.2.2. Subtractive library screen**

Two parallel subtractive cDNA libraries containing genes enriched for either mesoderm-specific or dorsal-lateral specific cDNAs was made according to the method described by Wang and Brown, 1991 (Casal and Leptin, unpublished). The library was plated and screened twice, first after the sixth round of subtraction, and second after the seventh round of subtraction. 5µg of dorsal-lateral specific cDNAs were digested with the restriction enzyme Eco RI according to conditions recommended by the manufacturer. 75ng of the digested DNA were ligated into 900ng of the cloning vector pBluescript II SK+ (Stratagene), previously linearized with Eco RI and treated with calf intestinal alkaline phosphatase (Boehringer Mannheim). The product of the ligation was transformed into XL-1 Blue supercompetent bacteria (Stratagene) in the following manner: The ligated DNA (10µl) was mixed with 100µl of supercompetent bacteria and allowed to incubate on ice for 30 minutes. The bacteria were heat shocked at 42°C for 3 minutes and topped with 1ml of LB medium. The bacteria were allowed to grow for 1h at 37°C on a shaker, and plated at a dilution so as to obtain an average of 600 colonies per 14.5 x 2 cm LB agar plate.

Duplicate colony lifts were made from each plate using Hybond N nylon membranes (Amersham). The colonies were allowed to grow for several hours at 37°C on fresh LB agar plates containing ampicillin until the colonies on each duplicate pair of filters were the same size in diameter. The plates were allowed to cool for one hour at 4°C and the bacteria were lysed and the DNA fixed onto the membranes according to the procedure described by Sambrook et al., 1989.

The filters were prewashed in 0.5% SDS, 0.1X SSC at 68°C for 1 hour and then prehybridized in Church and Gilbert hybridization solution (1% BSA, 0.5mM sodium phosphate pH 6.8, 50mM EDTA, 7% SDS) at 68°C for 1-2 hours. 1µg of the enriched cDNA pool from each library (dorsal-lateral specific and mesoderm specific) was labeled using the random primer method (Feinberg and Vogelstein, 1983). The appropriate denatured probe was added to the prehybridization solution and allowed to hybridize at 68°C overnight. The filters were washed at 68°C, 2 X 30' and 68°C, 1 X 30', and then exposed to autoradiographic film overnight.

All signals that appeared only on the set of filters probed with the enriched pool of dorsal-lateral specific cDNAs and not on the set of filters probed with the enriched pool of mesoderm-specific cDNAs were picked and grown overnight in 96 deep-well multititre plates (Beckman) in 1ml LB containing 50µg/ml ampicillin. Plasmid DNA was extracted using the alkaline lysis method (Sambrook et al., 1989). Dot blots of each clone were made in multiple copies on nylon membranes and the DNA fixed by



baking the filters at 80°C for 2h. Each membrane (which contained approximately 1ng of DNA from each dorsal-lateral clone) was hybridized to the radiolabeled Eco RI fragment of a particular clone using the same hybridization, labelling, and washing procedures as described above. The first ten clones (DL1-DL10) were used as probes and any of the remaining clones that cross-hybridized were noted down. A second set of 10 clones that did not cross-hybridize was then used to rescreen the dot blot filters, and so on until all cross-hybridizing fragments were accounted for. Each different clone from the first screen was radiolabeled and used to screen the library filters from the second screen in order to identify the new dorsal-lateral specific clones.

Each plasmid containing a dorsal-lateral specific cDNA fragment was digested with Eco RI and the insert separated from the vector using preparative TBE agarose gel electrophoresis. DNA was recovered from the gel using QIAEX beads (QIAGEN) and eluted in 20µl TE (10mM Tris pH7.5, 1mM EDTA).

### **2.2.3. DNA sequencing**

All sequences were carried out using double-stranded DNA as a template. The SK (5'-CTAGGTGATCAAGATCT-3') and KS (5'-CGAGGTCGACGGTATCG-3') primers were used for sequencing the plasmids containing the dorsal-lateral specific cDNA fragments obtained from the library. Sequencing reactions were performed according to the dideoxy chain termination method (Sanger et al., 1977). The reactions were loaded onto 6% Urea-Polyacrylamide gels and run on the Macrophor System (Pharmacia). Sequences were analyzed using the GCG software (University of Wisconsin) and the BLAST network service (NCBI).

### **2.2.4. Isolation of cDNA, cosmid and phage genomic clones**

cDNA clones were obtained by screening a 4-8 hr pNB40 *Drosophila* cDNA library (Brown and Kafatos, 1988). Cosmid clones were obtained by screening a cosmid library prepared by Tamkun et al., 1992. Phage genomic clones were obtained by screening a lambda DASH phage genomic library (Caudy et al., 1988). Probes were generated using the Eco RI fragment of a clone of interest, and the random primer method as described above.

### **2.2.5. Southern analysis using cDNAs as probes**

In cases where certain non-crosshybridizing clones gave the same expression pattern, each Eco RI fragment was separated from the vector using preparative TBE gel electrophoresis (1X TBE buffer : 45mM Tris/Borate, 1mM EDTA and 1-2% w/v agarose) and blotted onto a nylon membrane according to the Southern blot protocol

described in Sambrook et al., 1989. A cDNA from one of the clones was radiolabeled and allowed to hybridize to the other clones.

### 2.2.6. *In situ* hybridizations to whole mount embryos

Embryos between stages 5 and 10 were dechorionated with bleach, rinsed with tap water, and fixed at room temperature for 30' on a shaker in a 3.7% formaldehyde (PBS)/ heptane solution (1:2). The aqueous phase was removed and an equal volume of methanol was added to devitellinize the embryos. The embryos were rinsed several times in methanol and stored at -20°C until further use. 100ng of gel purified fragments were labeled overnight at room temperature with digoxigenin (Boehringer Mannheim) according to the manufacturer's instructions. The probes were precipitated in ethanol containing 5mM EDTA, 100mM LiCl, and 200µg/ml tRNA at -70°C for 1h, spun down for 15' at full speed in a microfuge and the pellet washed in 80% ethanol. The pellet was air dried and resuspended in 20 µl of TE. One tenth of the labeled probe was used per 100 µl of hybridization solution.

*In situ* hybridizations were performed using a slightly modified protocol of the one described by Tautz and Pfeifle, 1989. Fixed embryos were rehydrated in a methanol series of 70%, 50%, 30% methanol in PBT (0.1% Tween 20 in PBS), followed by 2 X 5' incubations in PBT. The embryos were refixed in 1ml 3.7% formaldehyde for 20' on a rotator and washed 3 X 5' with PBT. The embryos were incubated for 5' at room temperature in 1ml of Proteinase K 50µg/ml (Sigma Molecular Biology Grade) in PBT, followed immediately with 20µl of Glycine (100mg/ml). After the embryos had settled, the Proteinase K solution was removed and 1ml of Glycine (2mg/ml) in PBT was added. The solution was mixed, allowed to settle and the embryos washed 3 X 5' in PBT. The embryos were refixed and washed in PBT as described above, followed by a final wash in a 1:1 mixture of PBT:Hybridization solution (50% formamide, 5XSSC, 100ng/ml tRNA, 0.1% Tween 20). Embryos were prehybridized at 48°C for 1-4h in 100-200µl prehybridization solution (hybridization solution with 100ng/ml salmon sperm DNA). The denatured probe was added and allowed to hybridize overnight at 48°C. The next day, embryos were washed as follows: Hybridization solution 20' at 48°C, Hybridization solution:PBT (1:1) 20' at 48°C, and 3 X 20' PBT at room temperature. The embryos were incubated at room temperature for 1h with the Anti-DIG-alkaline phosphatase conjugated antibody (Boehringer Mannheim) in PBT (1:1000). The embryos were washed 3 X 5' in PBT, followed by a final wash in a 1:1 mixture of PBT:Staining buffer (100mM NaCl, 50mM MgCl<sub>2</sub>, 100mM Tris/HCl pH 7.5, and 0.1% Tween 20). The staining reaction was performed in 24-well plates in 1ml each of staining solution (1ml staining buffer with 4.5µl of NBT (75mg/ml in 70% dimethylformamide (DMF)) and 3.5µl of X-

phosphate (50mg/ml in 100% DMF). The staining reaction was stopped by washing several times with PBT.

### **2.2.7. Sectioning of embryos**

Stained embryos were dehydrated in an ethanol series of 30%, 50%, and 70% ethanol in PBS followed by 2 X 5' incubations each in 100% ethanol, 100% dehydrated ethanol, and dehydrated acetone. They were then transferred into a 1:1 mixture of dehydrated acetone and Araldite (Durcupan ACM, Fluka). Araldite was prepared by mixing 100ml of reagent A with 100ml reagent B, followed by 3.5ml and 2ml of reagent D and reagent C respectively. The mixture was stored as aliquots in 5ml syringes at -20°C until further use. The embryos were allowed to equilibrate into the acetone:Araldite mixture overnight in an open plastic dish until all the acetone had evaporated. The embryos were then embedded in fresh Araldite and oriented for sectioning. The embryos were allowed to settle to the bottom of the embedding blocks overnight at 4°C, reoriented and polymerized for 24-48h at 58°C. Serial cross-sections of 5µm, 10µm, or 20µm were made using a Reichert-Jung Supercut 2050 microtome. The sections were mounted in Araldite and photographed on a Zeiss Axiophot with Nomarski optics.

### **2.2.8. Analysis of cross-sections**

Mutant embryos were lined up against wild-type embryos of the same stage and subjected to serial cross-sectioning of 5µm, 10µm, and 20µm from anterior to posterior. Morphological criteria were used to distinguish various domains and to facilitate the identification of boundaries of gene expression. In certain cases, counting of stained cells on cross-sections proved impossible due to irregularities in cell morphology. This was also the case when staining was very weak and thick sections had to be made at the cost of cell resolution. Thus, transparent paper was used to trace cross-sections of mutant embryos. These were then overlaid onto photographs of cross-sections of wild-type embryos to visualize any shift in expression pattern. The average of cell counts of stained cells made on consecutive cross-sections of wild-type and mutant embryos were compared with one another. During early stages (between blastoderm stage and early gastrulation), mutants could not be distinguished from wild-type using morphological criteria. Thus, embryo counts were the only means of determining which embryos were mutant in cases where differences in expression patterns were observed.

Another problem arose when performing cell counts at the fully extended germ band stage. The width of different regions along the dorsal-ventral axis during

extended germ band no longer corresponds to the width of the respective primordia at the blastoderm stage. The widths also vary along the anterior-posterior axis of the embryo. This is due to cell intercalation that occurs during germ band extension. (Irvine and Wieschaus, 1994). Thus, it was useful to follow the development of these regions in embryos that do not extend their germ bands (or only to a very limited degree) like *hunchback knirps* (*hb kni*) double mutant embryos (*kni<sup>IID</sup>hb<sup>7M</sup>*). In these embryos one can accurately count the number of cells expressing a particular gene since all the cells along the embryonic circumference are evenly spread out and undergo the same cell shape changes as in wild-type. These mutant embryos were particularly useful for analyzing the gene expression patterns of many of the markers used during stage 8 and beyond. The cells of the ventral ectoderm appear enlarged as in wild-type of the same stage (stage 8). The dorsal ectodermal cells are smaller and cuboidal and the cells of the amnioserosa are flattened. Shortly after, cells of the ventral ectoderm begin to delaminate to form neuroblasts and acquire a slightly irregular appearance. The dorsal ectodermal cells become tall and columnar and form a continuous epithelium with the ventral ectoderm. The two regions can still be distinguished from one another however, because the border between the two is marked consistently by a kink. The amnioserosa cells have flattened by this stage and appears as a thin membrane. In wild-type embryos, the membrane is folded behind the head and flanks both sides of the germ band. In *hb kni* embryos, the amnioserosa forms a fold along the dorsal midline.



**CHAPTER 3**  
**RESULTS**

## RESULTS

### Subtractive cDNA library screen

As mentioned previously in the introduction, a subtractive cDNA library was screened in order to identify genes that are expressed in specific regions along the dorsal and lateral regions of the *Drosophila* embryo. After two rounds of screening, a total of 24 non-crosshybridizing clones were obtained (see Appendix A for numbers regarding the screen). Two clones turned out to be cDNA fragments of previously identified genes. One clone had an identical sequence to the *Drosophila* POU domain genes *pdm-1* and *pdm-2*, while the other turned out to be *tld*, one of the dorsal-ventral patterning genes (see Appendix B). The other clones appear to represent novel genes based on preliminary sequence analysis and *in situ* hybridization expression patterns.

The expression patterns of the other 22 clones are quite varied. Some can be detected as early as syncytial blastoderm, and remain as long as germ band extension or beyond. Several are expressed along the entire dorsal-lateral region and stop abruptly at the mesoderm boundary, while others are expressed more specifically, either as a thin strip of cells along the dorsal midline or as larger patches in various parts of the dorsal domain. Still others are expressed in the amnioserosa or in specific tissues in later embryonic stages. The various clones were categorized according to their expression patterns and subdivided into 5 groups as follows: 1) amnioserosa, 2) dorsal-lateral ectoderm, 3) tracheal system, 4) other late genes, and 5) known genes.

### Expression patterns of the dorsal-lateral clones

All stages and embryonic structures are as described by Campos-Ortega and Hartenstein (1985) unless otherwise noted. See figures 3.1 and 3.2 for summary of expression patterns. (Note: The expression patterns described below may not be absolutely final since it was later discovered that the 400 bp fragments used as probes did not always reveal the full expression pattern of the gene.)

### Clones expressed in the amnioserosa

#### *clone 20*

Clone 20 expression first emerges during early cellular blastoderm as a latitudinal stripe across the dorsal part of the embryo that lies just anterior to the region that will become the cephalic furrow. Thus, the embryo appears to be wearing a 'collar'. A dorsal posterior spot can also be detected during this stage. As cellularization progresses, a longitudinal stripe approximately 6 cells wide arises between the collar and the posterior spot. The emergence of the dorsal longitudinal stripe is coincident

with the refinement of the *zen* pattern and lies within the presumptive amnioserosa where *zen* is also expressed. By cellular blastoderm, expression intensifies, and the anterior-posterior stripe extends slightly more anterior than the collar. The overall expression pattern resembles that of a donkey's back. Hence, clone 20 will from now on be referred to as 'donkey-back'(*dkb*). *dkb* is confined to the primordium of the amnioserosa from the beginning, and is thus a good early marker for this region. The *dkb* pattern persists until late stage 8. By stage 9, the expression in the amnioserosa begins to fade, and expression in the tracheal pit anlage arises. This *de novo* expression initially appears as a series of spots surrounding the amnioserosa, but as the germ band retracts, expression extends dorsally and ventrally so that a series of stripes can be seen in the segmented region of the embryo.

### **Clone 14**

Clone 14 is expressed in a grainy pattern and first appears during ventral furrow formation as a large block within the dorsal-lateral trunk region of the embryo. By stage 9, expression is confined to the amnioserosa.

### **Clone 3**

Clone 3 expression only appears at stage 9 when the germ band has fully extended. Expression initially comes on in a small region adjacent to the opening of the posterior midgut on the dorsal side of the embryo. By stage 10 full expression appears in the amnioserosa. Staining is cytoplasmic and the squamous appearance of the amnioserosa cells is clearly visible. Expression in the amnioserosa persists until the end of dorsal closure. Clone 3 has an identical expression pattern to clones 26, b23, b67, and b144.

### **Clone b46 (*u-shaped-like*)**

Clone b46 is expressed in a similar fashion to the gene *u-shaped* (*ush*) (Ray,1993). *ush* acts downstream of *zen*, and is believed to play a role in the morphological differentiation of the amnioserosa. Expression comes on at cellular blastoderm in the  $\partial A$ , and in  $\partial_{14}19$ , making it wider than *zen* expression. This pattern is maintained until at least as far as extended germ band.

## **Clones expressed in the dorsal-lateral ectoderm**

### **clone 31**

Clone 31 has the same expression pattern as the *Drosophila* gene *crumbs* (*crb*) which encodes an EGF-like protein and is involved in the development of the epithelia (Tepass et al., 1990). Transcripts are present in the blastoderm embryo in all cells of the ectoderm, but not in mesodermal cells. By stage 7, clone 31 is also expressed in

the amnioproctodeal invagination and in other regions of the embryo where folds occur, such as the cephalic furrow. By stage 11 staining can be seen in the stomodeum, the developing hindgut, and the malphigian tubules. Staining in the posterior midgut primordium, a structure that arises from the amnioproctodeal invagination, is also present, but disappears shortly before it loses its epithelial character. Like *crb*, clone 31 expression is localized to the apical surface of epithelial cells and is suppressed by *sna* in the mesodermal anlage (data not shown). Clone 31 has an identical pattern to clones 36, b120, and b135.

### ***Clone b93***

Clone b93 is expressed in a similar fashion as the neurogenic gene *Delta* (*Dl*) (Haenlin et al., 1990). Ectodermal staining covers most of the embryo except for the anterior and posterior poles, where expression is completely absent. Mesodermal staining is much more faint than in other regions. By fully extended germ band, b93 is expressed in a metameric fashion in the ectoderm.

### ***Clone 15***

The expression of clone 15 is similar to *crb* except that the ectodermal expression occurs in a graded fashion with the strongest expression appearing in the most dorsal regions. A gap in expression between the trunk region and the anterior pole can also be seen. At the posterior pole, staining is also absent, particularly in the amnioproctodeal invagination, where *crb* is normally expressed.

### ***Clone 19***

Clone 19 is expressed during retracted germ band in the dorsal epidermal primordium. It is absent in the amnioserosa and coincides with *pnr* expression during this stage.

### ***Clone b39***

Clone b39 is expressed in the entire dorsal and lateral epidermis and is absent in the ventral ectodermal region overlying the ventral nerve cord.

## **Clones expressed in the tracheal system**

### ***Clone b50***

Clone b50 is expressed in the tracheal placodes. Staining can be detected at stage 10 along the dorsal-lateral region of the extended germ band ( $\partial_{1411}$ ) as a series of spots along each side of thoracic segments 1 and 2, and abdominal segments 1-8. Staining is also present in the prothorax, where the anterior spiracle, the anterior

opening of the tracheal tree develops. Expression in the posterior spiracle however, is much more faint. This pattern is maintained until shortly before retracted germ band (stage 12) where expression is seen to extend dorsally and ventrally as the invaginated tracheal pits elongate to form dorsal and ventral stems.

### ***Clone33***

Clone 33 transcripts are not detected until stage 12 when the germ band has begun to retract. Expression is exclusively in the tracheae and the characteristic branched pattern can be seen. Expression in the tracheal system can still be detected at stage 15, but whether expression continues in later stages has not been determined.

### ***Clone 37***

In contrast to clone 33, clone 37 is expressed in the ectodermal region surrounding the tracheal pits and the posterior spiracle. Transcripts also appear earlier than clone 33 (stage 11), and additional transcripts are present in the clypeolabrum.

## **Genes expressed during later stages**

### ***Clone 41***

Clone 41 is initially expressed in the proctodeum, and in the stomodeum at lower levels. By stage 14 expression comes on in the posterior spiracles and on the ventral side in a pattern that exactly matches the ventral denticle belt pattern.

### ***Clone b136***

Clone b136 only appears at stage 15 and is expressed in the pharynx, esophagus, ventral ectoderm, the dorsal-lateral trunk, and the posterior spiracles.

## **Known genes**

### ***Clone 12 (POU domain)***

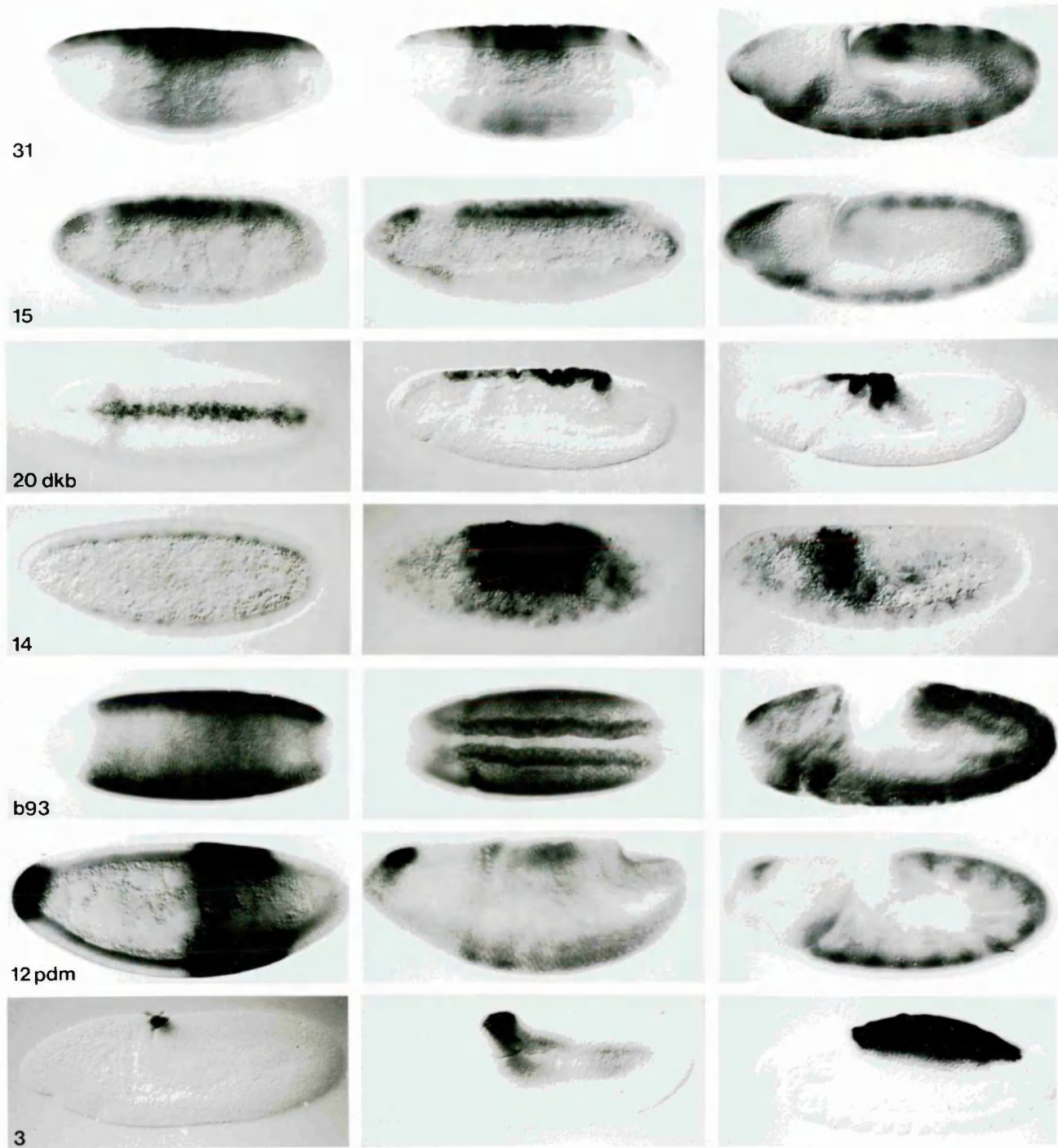
Clone 12 expression is identical to the expression of *pdm-1* and *pdm-2*. Transcripts are first detected during cellular blastoderm (stage 5) as two broad stripes in the abdominal segment primordia, and as a spot in the anlage of the clypeolabrum. By early extended germ band (stage 10), clone 12 is expressed in the tracheal placodes as well as the neurogenic region, with the exception of the ventral midline (Lloyd and Sakonju, 1991).

### ***Clone b29 (tolloid)***

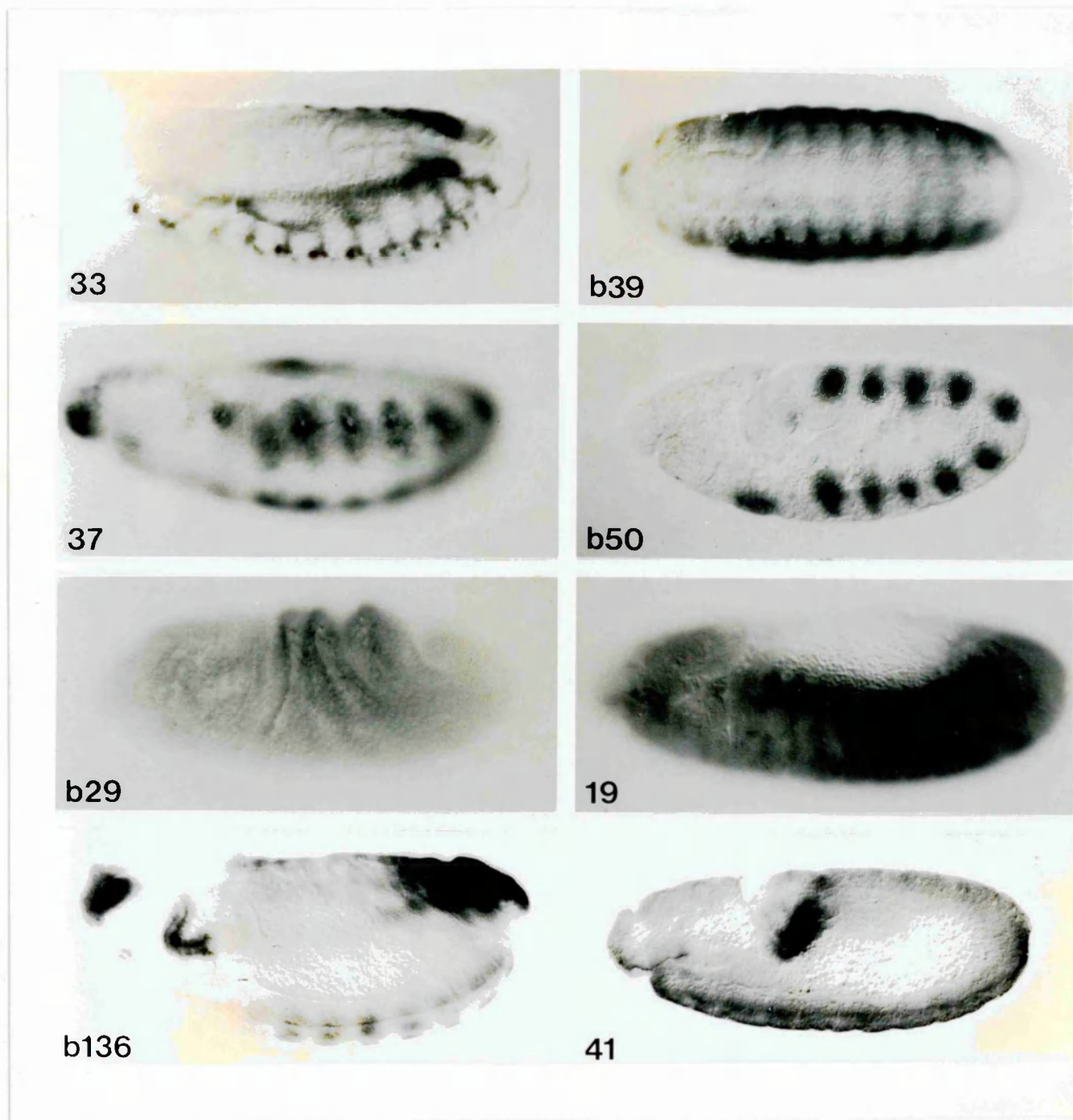
Despite the fact that the b29 sequence is identical to the *tld* sequence, we saw only some aspects of the reported expression pattern. By nuclear cycle 13, *tld* is expressed



over the dorsal 40% of the embryo circumference, with the exception of the pole cells. By cellularization, a reduction in staining of cells is located at each pole, as well as within the dorsalmost 10-20% of the embryo circumference. By late stage 8, expression can be seen in patches of cells that will become part of the gnathal segments, as well as in more posterior segments (Shimell et al., 1991). Although some weak dorsal epidermal staining for b29 was detected, additional staining in the amnioserosa was also present. Since *tld* is not normally expressed in the amnioserosa, the staining in this region as detected by the b29 probe is most likely non-specific. One possible reason for this discrepancy is the fact that the DNA probes consisting of 400 bp fragments were used for the *in situ* hybridizations. Hence, such small fragments may not give the full expression pattern. This may also explain why a number of genes expressed beyond stage 10 were isolated from the dorsal-lateral screen despite the fact that only embryos between stages 5 and 10 were used to make the library.

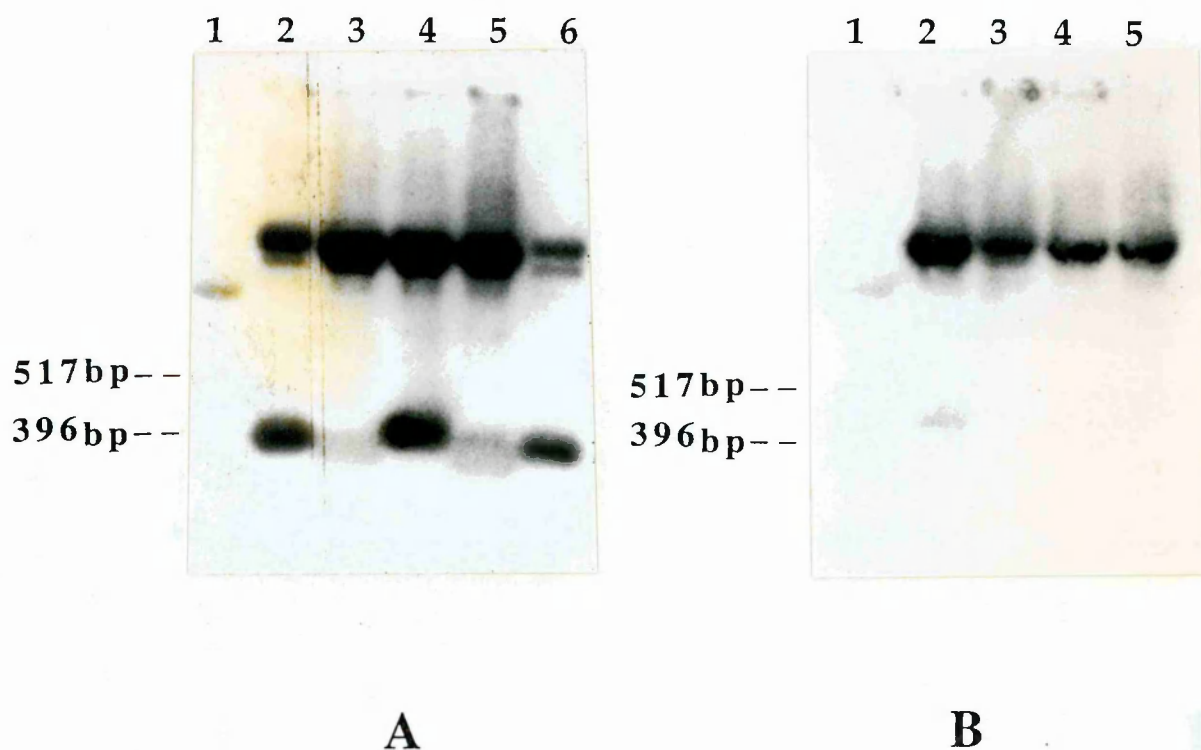


**Figure 3.1. Expression patterns of select dorsal-lateral clones.** *In situ* hybridization patterns of select dorsal-lateral specific genes on whole mount wild-type embryos. The expression pattern of each gene is shown at three different stages. From left to right in rows 1-6: Stage 5 (cellular blastoderm), Stage 6 (ventral furrow), and Stage 9 or 10 (extended germ band). From left to right in row 7: Stage 9 (extended germ band), Stage 10 (extended germ band), Stage 13 (retracted germ band). Clone numbers are on the lower left corner of the first embryo in each row. In most cases, dorsal is up and anterior to the left. Clone 31 (row 1): Expression in the entire dorsal-lateral epidermis. Clone 15 (row 2): Expression also in the entire dorsal-lateral epidermis but with stronger expression in dorsalmost part of embryo. Clone 20 (row 3): Expression in the presumptive amnioserosa and in a dorsal-lateral stripe just anterior to the cephalic furrow. Clone 14 (row 4): Grainy expression mainly in the dorsal-lateral trunk, then in the amnioserosa during fully extended germ band. Clone b93 (row 5): Expression in the dorsal-lateral epidermis with the exception of the poles. Clone 12 (*pdm*) (row 6): Expression in the clypeolabrum and two broad bands in the trunk. Tracheal pit staining in later stages. Clone 3 (row 7): Expression only in the amnioserosa.



**Figure 3.2. Expression patterns of dorsal-lateral clones expressed relatively late.** *In situ* hybridization patterns of select dorsal-lateral specific genes on whole mount wild-type embryos. Left column from top to bottom: Clone 33: Expression in the tracheae. Clone 37: Expression in the clypeolabrum and in the ectodermal region surrounding the tracheal pits. Clone b29 (*tld*): Expression in the dorsal-lateral epidermis. Clone b136: Expression in the pharynx, esophagus, ventral ectoderm, dorsal-lateral trunk, and the posterior spiracles. Right column from top to bottom: Clone b39: Late expression in the dorsal-lateral ectoderm. Clone b50: Initial expression in the tracheal placodes, then in the tracheal pits. Clone 19: Expression in the dorsal ectoderm just below the amnioserosa. Clone 41: Faint expression in the proctodeum and stomodeum with more intense expression in the posterior spiracles, and later in the denticle belts.





**Figure 3.3. Southern blots of cDNA 3 and cDNA 31 to other similarly expressed clones.** Left figure (A): cDNA 3 used as probe towards Eco RI cDNA fragments of non-cross-hybridizing dorsal-lateral clones with the same expression pattern. Top bands at 3kb represent pBluescript II SK+. Lanes 1-6: Molecular weight marker, clone 3, clone 26, clone b23, clone b67, and clone b144 respectively. Right figure (B): cDNA 31 used as probe towards Eco RI cDNA fragments of hybridizing dorsal-lateral clones with the same expression pattern. Lanes 1-5: Molecular weight marker, clone 31, clone 36, clone b120 and clone b135 respectively.

### **Southern Blot hybridization of cDNA 3 and cDNA 31 to other similarly expressed clones**

Since several different clones were obtained from the dorsal-lateral library that gave identical expression patterns, it was possible that these clones were non-overlapping fragments of the same gene. Therefore, cDNAs for clone 3 (*amnioserosa*) and clone 31 (*crb*-like) were obtained by screening a cDNA library from 4-8 hr embryos. The cDNAs were then labeled radioactively and used as probes on Southern blots containing all other similarly expressed clones (See figure 3.3). cDNA 3 hybridized to clones b23 and b144, but not to clones 26 and b67. cDNA 31 on the other hand, did not hybridize to any of the other *crb*-like clones.

### Sequence analysis of dorsal-lateral clones

Nucleotide sequences up to 200 bp were obtained for a number of clones (see Appendix B). Sequences were initially compared on the nucleotide level to sequences in the GenBank and EMBL databases using the GCG FASTA program. The sequences were then compared to the GenBank, GenBank updates, EMBL, and EMBL updates nucleotide sequence databases using the BLAST network service (NCBI). The nucleotide sequences were also translated in all six frames and compared to the SWISS-PROT, PIR, GenPept, and GenPept updates peptide sequence databases using the BLAST network service (NCBI). As mentioned earlier, clone b29 shared 100% homology with the *tld* sequence, while clone 12 shared 100% homology with the *Drosophila* POU domain sequence. Clone 31 was found to share 70% similarity with the *crb* protein in a region partly overlapping one of the EGF repeats of *crb*.

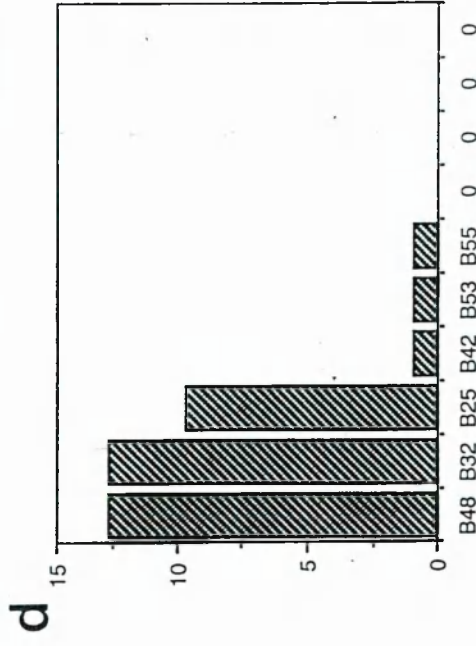
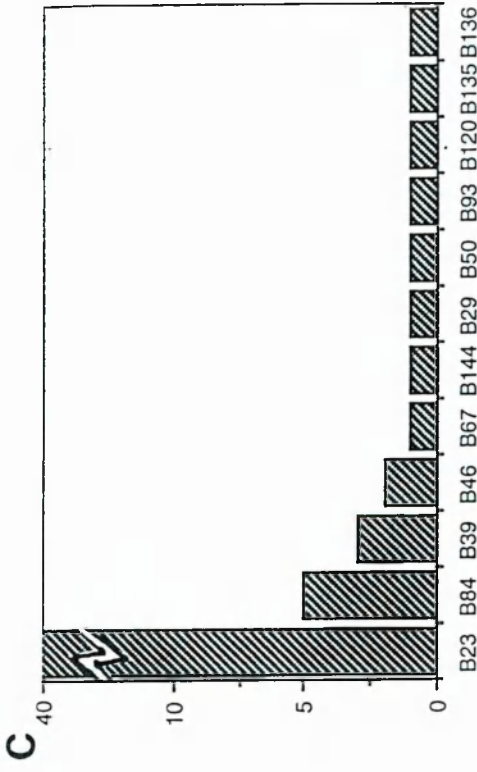
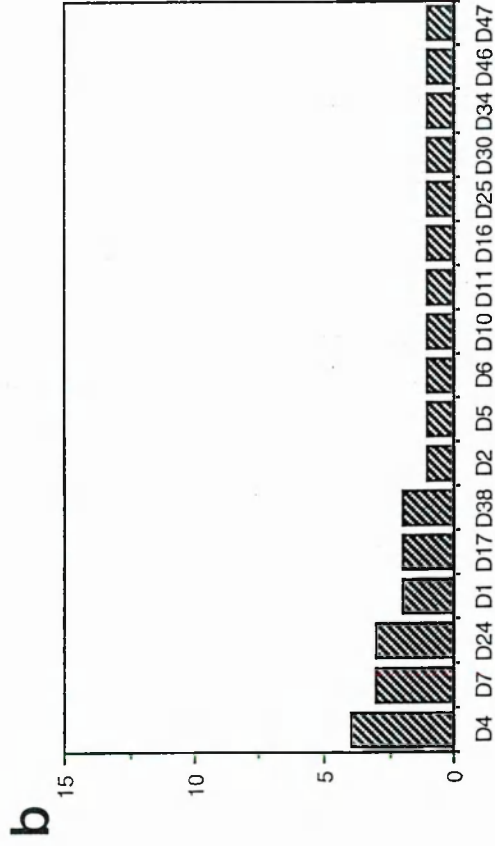
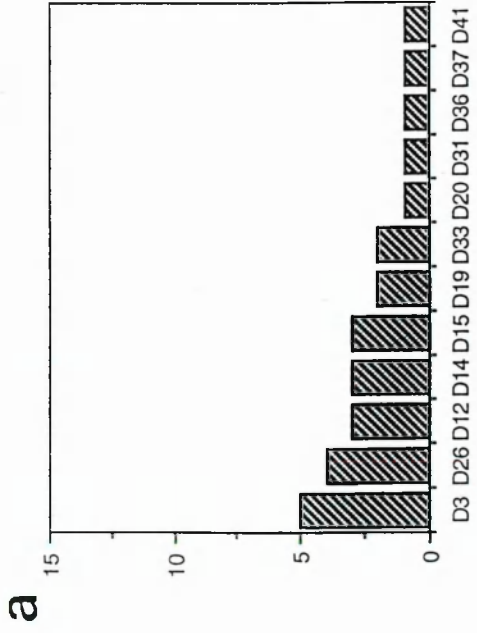
Clone b93 possessed 67% similarity with the neurogenic gene *Delta* on the nucleotide level. However when the b93 sequence was translated and aligned with the *Delta* protein sequence, only the first five amino acids were identical, and then diverged. If a frameshift in the b93 sequence is made by the addition of a random nucleotide just before the sequences diverge, then one can obtain as high as 67% similarity between the two sequences on the amino acid level. Therefore, b93 and *Delta* are not the same gene but possibly members of the same family. No significant homologies for any of the other clones were obtained.

### Discussion

The numerical results of the first and second screens of the dorsal-lateral library are presented in Appendix A. After screening the cDNA pool from the sixth round of subtraction, 7.2% of the screened clones were dorsal-lateral specific (as defined by their hybridization to only the dorsal-lateral probes). When a second screen on the cDNA pool from the seventh round of subtraction was performed, 10 % were dorsal-lateral specific. Thus, there was not a significant difference between the sixth and seventh round of subtraction.

If we assume that the clones that did not give a visible expression pattern are truly non-specific, then there is a substantial decrease in the number of non-specific clones obtained in the second screen compared to those obtained in the first. 50% of the clones from the first screen that hybridized to the dorsal-lateral probes alone did not give an expression pattern, as opposed to 10% of the clones in the second screen. (see figure 3.4, b and d)

Graphs illustrating the representation frequency of each individual clone are shown in figure 3.4 (next page). It is unlikely that the screen has reached saturation since the majority of clones were identified only once.



**Figure 3.4. Graphs illustrating the representation frequency of each individual dorsal-lateral clone.** Y axis represents the number of times a clone was isolated during the screen. X axis represents each individual clone by number (D=1st screen, B=2nd screen). (A) Graph illustrating the frequency of each clone from the 1st screen that gave a specific expression pattern. Five out of the twelve clones were only identified once. (B) Graph illustrating the frequency of each clone from the 1st screen that did not give a specific expression pattern. More than half were identified only once. (C) Graph illustrating the frequency of each clone from the 2nd screen that gave a specific expression pattern. Eight out of twelve were only identified once. Clone b23 was isolated 41 times. (D) Graph illustrating the frequency of each clone from the 2nd screen that did not give a specific expression pattern. Half of the non-specific clones were identified a significant number of times while the other half were only identified once.



Clone No.	Expression Pattern	Cross-hybridizing Clones	Non-crosshybridizing Clones (same pattern)	cDNA, phage clone or cosmid clone
3	amnioserosa	8, 21, 39, 52	26, 29, 49, 53	1
26	amnioserosa	29, 49, 53	8, 21, 39, 52	nx cDNA 3 (9p)
12	POU domain	23, 42	-	-
14	dorsal ectoderm	27, 28	-	-
15	d-v gradient	44, 54	-	1c, 3co
19	dorsal epidermis	9	-	9p
20	'donkey-back'	-	-	4p
31	<i>crbs</i> -like (epidermis)	-	36	2c, 2co
36	<i>crbs</i> -like (epidermis)	-	31	nx cDNA 31 (19p)
33	trachea	40	-	-
37	tracheal pits	-	-	-
41	vent. denticle belts	-	-	-
b23	amnioserosa	36 other clones	b67, b144	x cDNA 3
b67	amnioserosa	-	b23, b144	x phage clone 26, nx cDNA 3
b144	amnioserosa	-	b23, b67	x cDNA 3
b29	<i>tlid</i> (dorsal ectoderm)	-	-	-
b39	dorso-lateral epidermis	63, 88	-	-
b46	<i>ush</i> -like (amnioserosa)	43	-	-
b50	tracheal placode	-	-	-
b93	<i>DI</i> -like	-	-	1p
b120	<i>crbs</i> -like (epidermis)	-	b135	nx cDNA 31, nx phage 36 (3co)
b135	<i>crbs</i> -like (epidermis)	-	b120	nx cDNA 31, nx phage 36 (4 co)
b84	POU domain	-	b114, b121, b141, b125	-
b136	pharynx, esophagus	-	-	-

**Figure 3.5. Summary table of all the clones obtained from the dorsal-lateral screen. From left to right: Column 1: List of all dorsal-lateral clones obtained from the first and second screen that gave a specific expression pattern (b=second screen); Column 2: corresponding expression pattern of each clone; Column 3: cross-hybridizing clones; Column 4: non-crosshybridizing clones; Column 5: number of phage genomic, cosmid or cDNA clones obtained. x= crosshybridizes to, nx= does not cross-hybridize to, c=cDNA, p=phage, co=cosmid.**

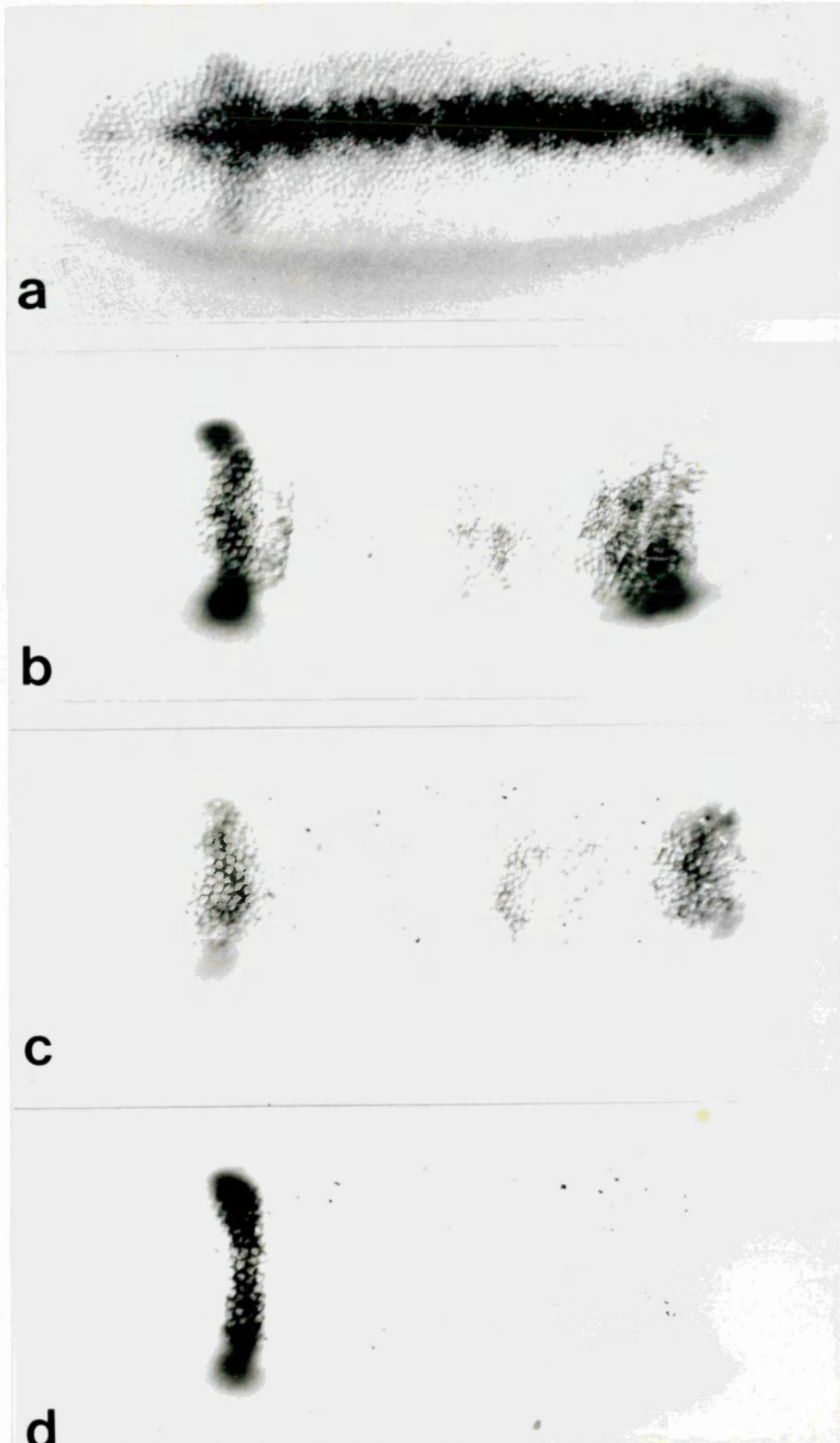
## Effects of zygotic ventralizing mutants on the expression of genes expressed in the amnioserosa

Two clones obtained from the dorsal-lateral library were utilized as markers for the amnioserosa region. *dkb* was chosen as a marker for the amnioserosa primordium ( $\partial A$ ), while clone 3 was chosen as a marker for the amnioserosa proper. In *dpp*, *tld*, *scw*, and *zen* mutants, the dorsal longitudinal stripe of *dkb* is absent. However, in *srw* and *tsg* mutants, small patches of faint staining can still be detected. Interestingly, the staining appears only in the posterior abdominal region and not in the thoracic region of the embryo. This patchy staining is present at cellular blastoderm when a solid line is already present in wild-type embryos. The patches can be detected until early gastrulation and then they decay.

The collar and the posterior spot are also affected to varying degrees among the various mutants. In *tld* and *dpp* embryos, the posterior spot is absent and only the collar remains. In *scw* mutants, only the collar is present in the blastoderm. However, during early gastrulation to early extended germ band, expression in the posterior end reappears. In *srw* and *zen* mutants, both the collar and the posterior spot are present, but are much more reduced in *zen* mutant embryos.

In *dpp*, *tld*, *scw*, and *zen* embryos, the expression of clone 3 is entirely absent. This is expected since the entire amnioserosa is missing in these mutants. In *srw*, *sog* and *tsg* embryos however, clone 3 expression is still present, but reduced. The cells that express clone 3 also possess the characteristic squamous appearance of amnioserosa cells, suggesting that some amnioserosa cells are still present in these mutant embryos. However, expression is transient since in older mutant embryos, staining disappears at a time when it would normally still be present in wild-type.

In *srw* mutant embryos, the initial *zen* pattern is established but rapidly degrades before the time when it would normally refine. In *sog* and *tsg* mutant embryos, the initial pattern is maintained, but never refines (Ray, 1993). These data reveal that the presence of amnioserosa cells in these mutants is not due to the persistence of *zen* expression since *zen* is not maintained in *srw* mutants. However, the presence of amnioserosa cells suggests that even a short pulse of *zen* expression is sufficient to specify a few cells to acquire the amnioserosa fate. Thus, some amnioserosa cells are still present and express genes normally expressed in these types of cells.



**Figure 3.6. Pattern of early *dkb* expression on zygotic ventralizing mutants.**

*In situ* hybridization pattern of *dkb* on whole mount wild-type and mutant embryos. All embryos are at the cellular blastoderm stage and photographed from the dorsal side. A-D: wild-type, *srw*, *tsg*, and *dpp* respectively. The collar is present in all mutants. Faint staining is present in *srw* and *tsg* embryos between the collar and posterior spot. The expression pattern of *dkb* in *tld* and *scw* mutants is identical to expression in *dpp* mutants.



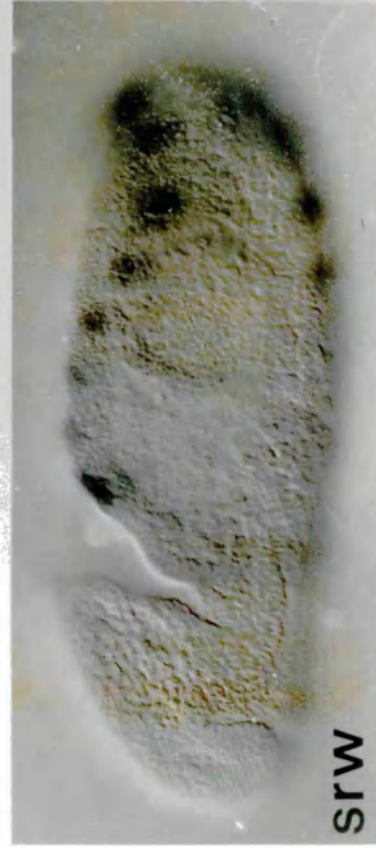
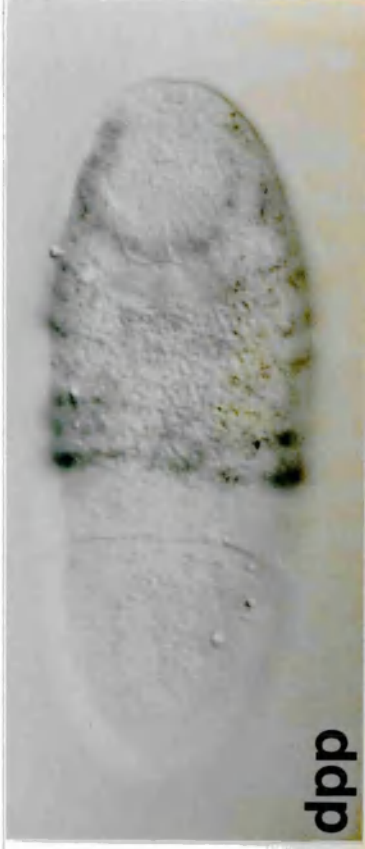
## Effects of zygotic ventralizing mutants on the expression of genes expressed in the dorsal epidermis

Clone b50 was chosen as a marker for the dorsal ectoderm because of its expression around the tracheal placodes, embryonic structures that have been mapped to  $\partial_{1411}$  (Foe, 1989). As mentioned previously, b50 is expressed as a series of spots running along each side of the thoracic and abdominal segments. Initial expression can be detected by stage 9, and is restricted to the region just above the dEp/VE boundary. By stage 10, expression appears to extend into the dorsal part of  $\partial_{14N}$ . However, comparisons between cross-sections of b50 expression at this stage and neuroblast staining as visualized by *sna* RNA staining reveals that the two never truly overlap, but rather alternately occupy the same region. In other words, the third row of neuroblasts which defines the dorsal most boundary of the neurogenic region is not arranged in a straight line. Instead, the neuroblasts are arranged more in a 'scallop' fashion, with the more dorsal tracheal placodes 'invading' regions of the ventral neurogenic region. This raises the question as to whether the dEp/VE boundary can still be defined as a boundary.

In *dpp*, *itd* and *scw* embryos, b50 expression extends all the way to the dorsal midline. In *srw* embryos, b50 expression is identical to that of wild-type. In *sog* mutant embryos, b50 expression also appears normal. Notably, the late expression pattern of *dkb* is also specific to the dorsal epidermis, and is affected in the same manner as b50 in all of the zygotic ventralizing mutants.

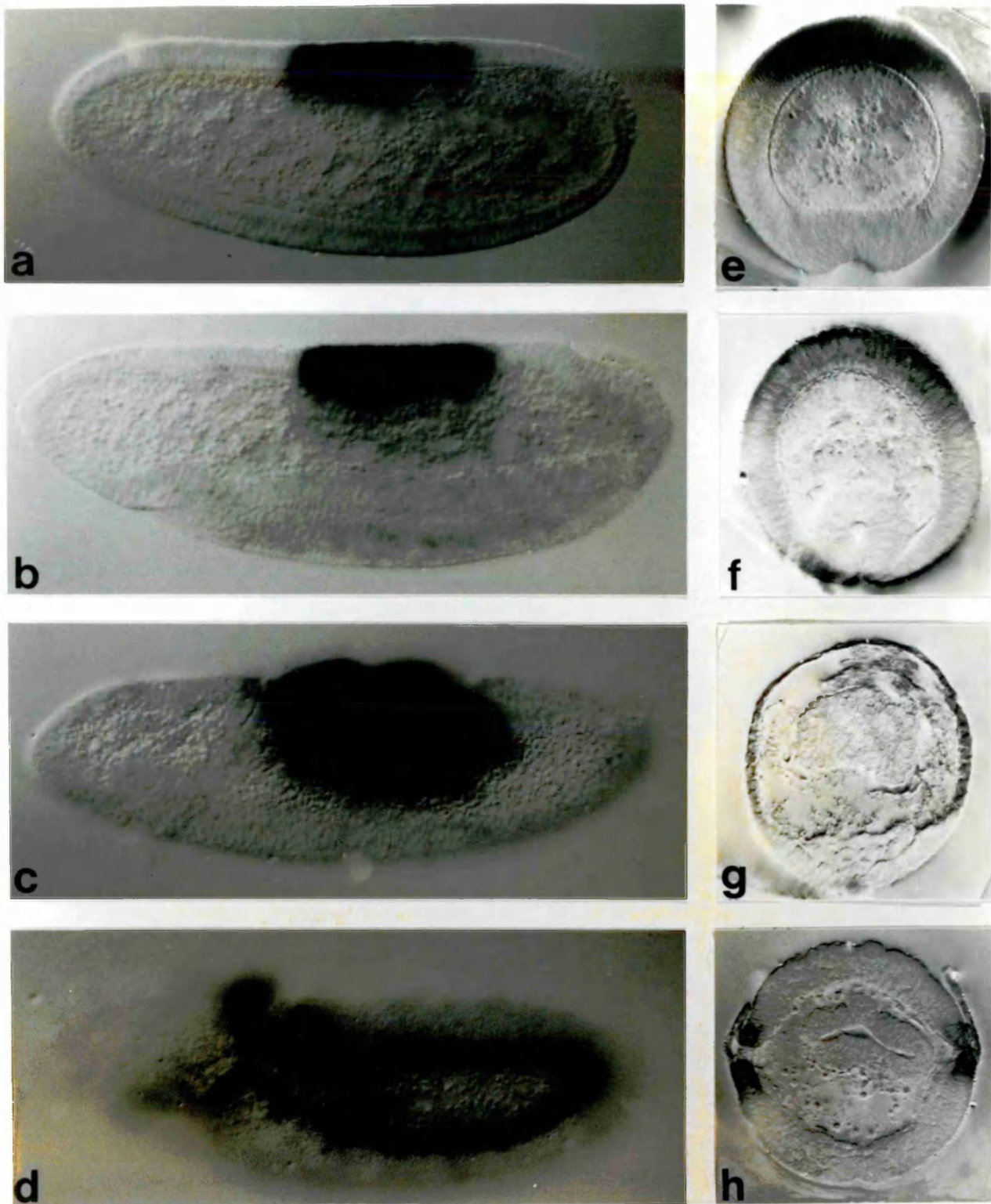
Because b50 is expressed relatively late, another gene, *pannier* (*pnr*), was chosen as an additional marker for the dorsal ectoderm. *pnr* is a transcription factor with high homology to vertebrate GATA-1 (Romain et al., 1993; Winick et al., 1993). Embryos mutant for this gene possess a large hole on their dorsal side (Jürgens, et al., 1984). *pnr* expression can be detected as early as syncytial blastoderm as two faint stripes perpendicular to the anterior-posterior axis in the dorsal abdominal region of the embryo. By cellular blastoderm, *pnr* expression intensifies, revealing two additional stripes, and by the onset of gastrulation, up to six stripes can be detected within the trunk region. Counts of *pnr* expressing cells on cross-sections at this stage average to 32. By stage 7, when the mesoderm has fully invaginated, *pnr* expression appears to extend further ventrally due to gastrulation movements. In addition, stripes fuse into a single block since the dorsal cells are pushed closer together due to germband extension. It is only during stage 8 that *pnr* is clearly seen to be restricted to cells within  $\partial_{1411}$ ,  $\partial_{1419}$ , and  $\partial A$ . Thus, the ventralmost boundary of *pnr* coincides exactly with the boundary between dorsal and ventral ectoderm. *pnr* continues to be

expressed within  $\partial_{14}11$  and  $\partial_{14}19$  throughout germ band extension, but progressively fades from the amnioserosa until it completely disappears from this region by stage 11.



**Figure 3.7. Pattern of b50 expression in zygotic ventralizing mutants.** *In situ* hybridization pattern of b50 on whole mount wild-type and mutant embryos. Anterior to the left and dorsal up except for the *dpp* embryo which is photographed from the dorsal side. All embryos are at the fully extended germ band stage. In wild-type embryos, b50 expression first appears during extended germ band in a series of spots surrounding the tracheal placodes. This region has previously been mapped to the dorsal epidermis and corresponds to  $\partial 1411$  (Foe, 1989). In *dpp*, *tld* and *scw* embryos, b50 expression extends all the way to the dorsal midline. In *sog* embryos, expression is the same as in wild-type. In *srw* embryos, expression appears to have shifted dorsally in a wave-like manner. Expression also appears somewhat weaker. However, this may simply be due to the overall weak staining in this batch of embryos.





**Figure 3.8. *In situ* hybridization pattern of the *pnr* gene on wild-type embryos at different stages of development.** Anterior to the left and dorsal up in the case of whole mounts. (A) lateral view of a stage 5 (blastoderm) embryo. Several stripes extend over the dorsal part of the trunk. (E) cross-section of a stage 6 (ventral furrow) embryo. Staining present in approximately the dorsal third of the embryonic circumference. (B) lateral view of a stage 7 embryo. Expression is slightly more intense. (F) cross-section of an embryo at the same stage as in B. Expression appears to extend further ventrally. This is most likely due to an expansion of the cells due to the invagination of the mesoderm on the ventral side. (C) stripes have fused and expression extends further ventrally due to cell intercalation caused by germ band extension. (G) cross-section of an embryo at the same stage as in C. Expression in the dividing round cells of the dorsal epidermis and the flattened cells of the amnioserosa. (D) lateral view of a stage 10 (extended germ band) embryo. Expression is now absent in the amnioserosa. (H) cross-section of an embryo at the same stage as D. Some expression can still be seen in the amnioserosa.

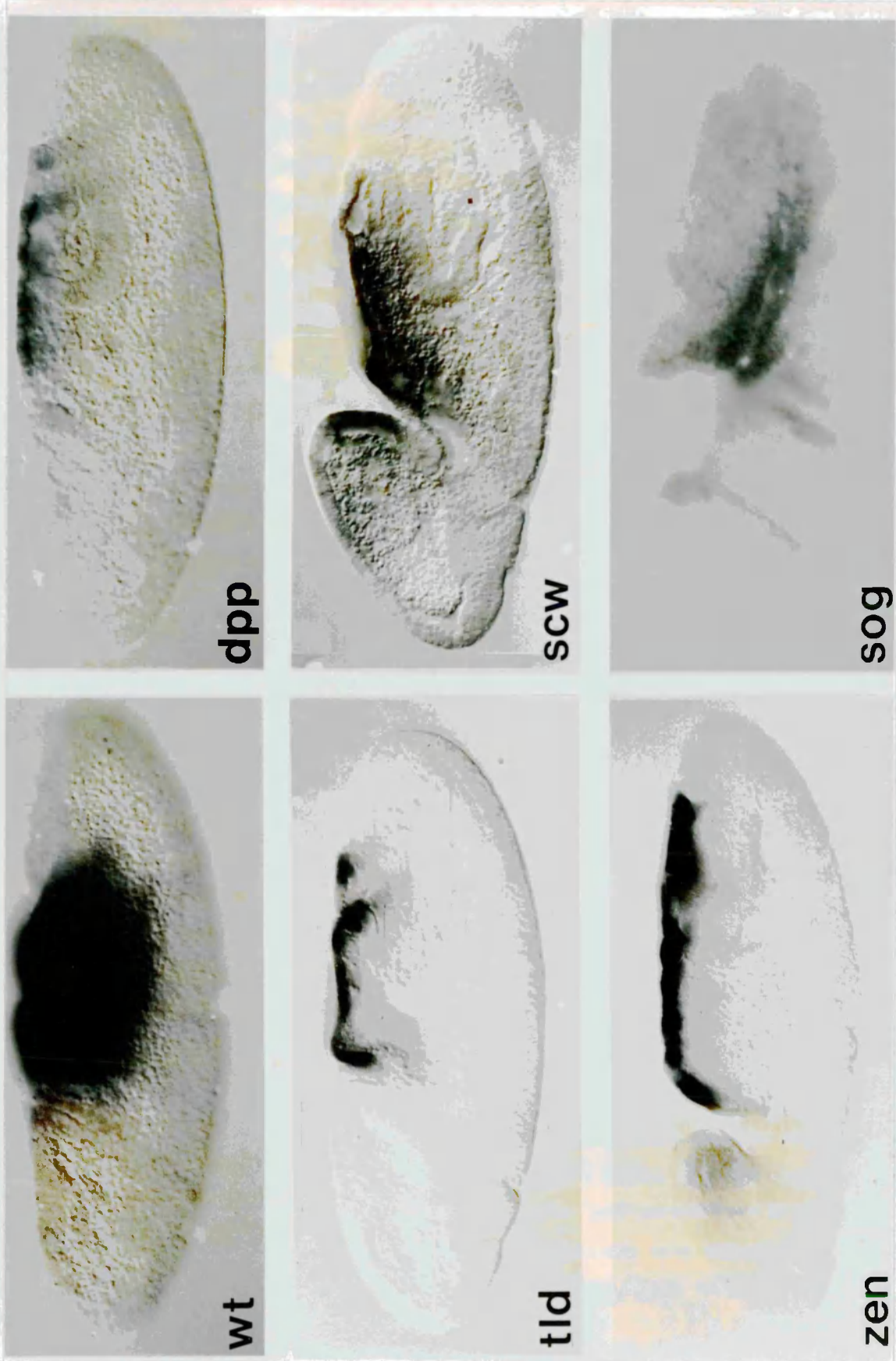
Analysis of *pnr* expression on *dpp* embryos revealed that *pnr* is still present in these embryos at early stages. When a collection of cellular blastoderm embryos which contained 25% embryos homozygous for the *dpp* mutation were analyzed for *pnr* expression, all had *pnr* staining. However, 6 out of 28, or 21.4 % had much weaker staining than the rest. Since this number is very close to 25%, I interpret these to correspond to the *dpp* mutant embryos. *pnr* persists in *dpp* embryos until about stage 6 where it is rapidly degraded. However, by fully extended germ band, no further staining can be detected in *dpp* embryos. Based on these data, it appears that *dpp* is not required by *pnr* for activation, but rather for maintenance of expression.

In *tld* and *scw* embryos, *pnr* expression is still present. However, expression is narrower and becomes less intense during the fully extended germ band stage. The narrow expression is more likely due to the slow but constant decay of *pnr* expression in these mutant embryos, since in earlier stages, *pnr* expression in mutant embryos cannot be distinguished from expression in wild-type.

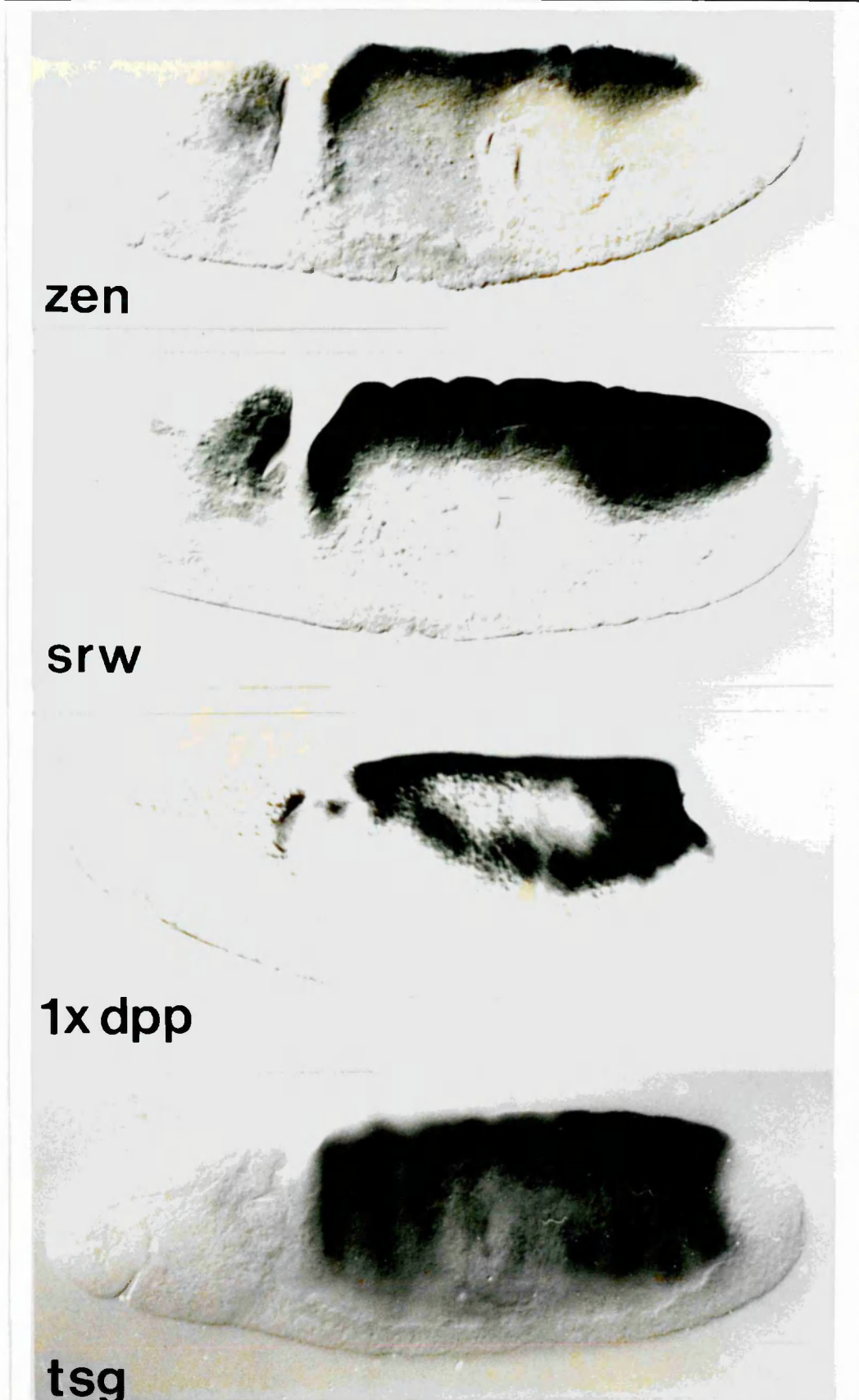
In *zen* embryos, *pnr* expression is the same as in wild-type during cellular blastoderm (i.e. encompassing the presumptive amnioserosa ( $\partial A$ ), the presumptive dorsal edge ( $\partial_{1419}$ ), and the presumptive dorsal epidermis ( $\partial_{1411}$ )). However, by stage 10, the two late *pnr* stripes ( $\partial_{1411}$ ) appear fused within a narrow region along the dorsalmost part of the embryo in *zen* mutants. In *tsg* embryos, *pnr* is expanded all the way to the dorsal midline at the same stage that the two late  $\partial_{1411}$  stripes fuse on the dorsal side in *zen*. The most ventral boundary of *pnr* is the same as in wild-type, making the expression of *pnr* in *tsg* embryos much wider than in *zen* embryos and arguing against a shift of the *pnr* expressing region to a more dorsal domain. In addition, *pnr* expression is interspersed with small irregular patches of cells with no *pnr* staining. These most likely correspond to the cells that still express amnioserosa markers such as *dkb* and clone 3.

*pnr* is expressed in a similar fashion in *srw* embryos as it is in *tsg* embryos, except that expression appears more in a wave-like fashion, with the expression in the more anterior part of the embryo being slightly higher than the expression in the more posterior regions. *pnr* expression on *srw* and *zen* embryos is very similar to that of 1x *dpp* embryos. This is not unexpected since only the amnioserosa is missing in these embryos. In *zen* embryos however, the region where *pnr* is expressed appears narrower than in *srw* and 1x *dpp* embryos.





**Figure 3.9. Pattern of *pr* expression in zygotic ventralizing mutants.** *In situ* hybridization pattern of *pr* on whole mount wild-type and mutant embryos. Anterior to the left and dorsal up. The wild-type, *dpp* and *tld* embryos are at stage 8 while the *scw*, *zen* and *sog* embryos are older (extended germ band). In the wild-type embryo, *pr* expression is present in the amnioserosa and dorsal epidermis ( $\partial A$  and  $\partial_{1,4}11$ ). In *dpp*, *tld* and *scw* embryos, *pr* expression is still present, but soon starts to degrade after the stages shown here. Cross-sections also reveal that some cell death occurs on the dorsal side in these later stages and may account for the disappearance of *pr* expression in these embryos. In *zen* embryos, the two longitudinal stripes of *pr* expression fuse at the dorsal midline. In *sog* embryos, expression expands ventrally into the dorsal part of the neurogenic region. This expansion can already be seen in cross-sections of *sog* blastoderm embryos.



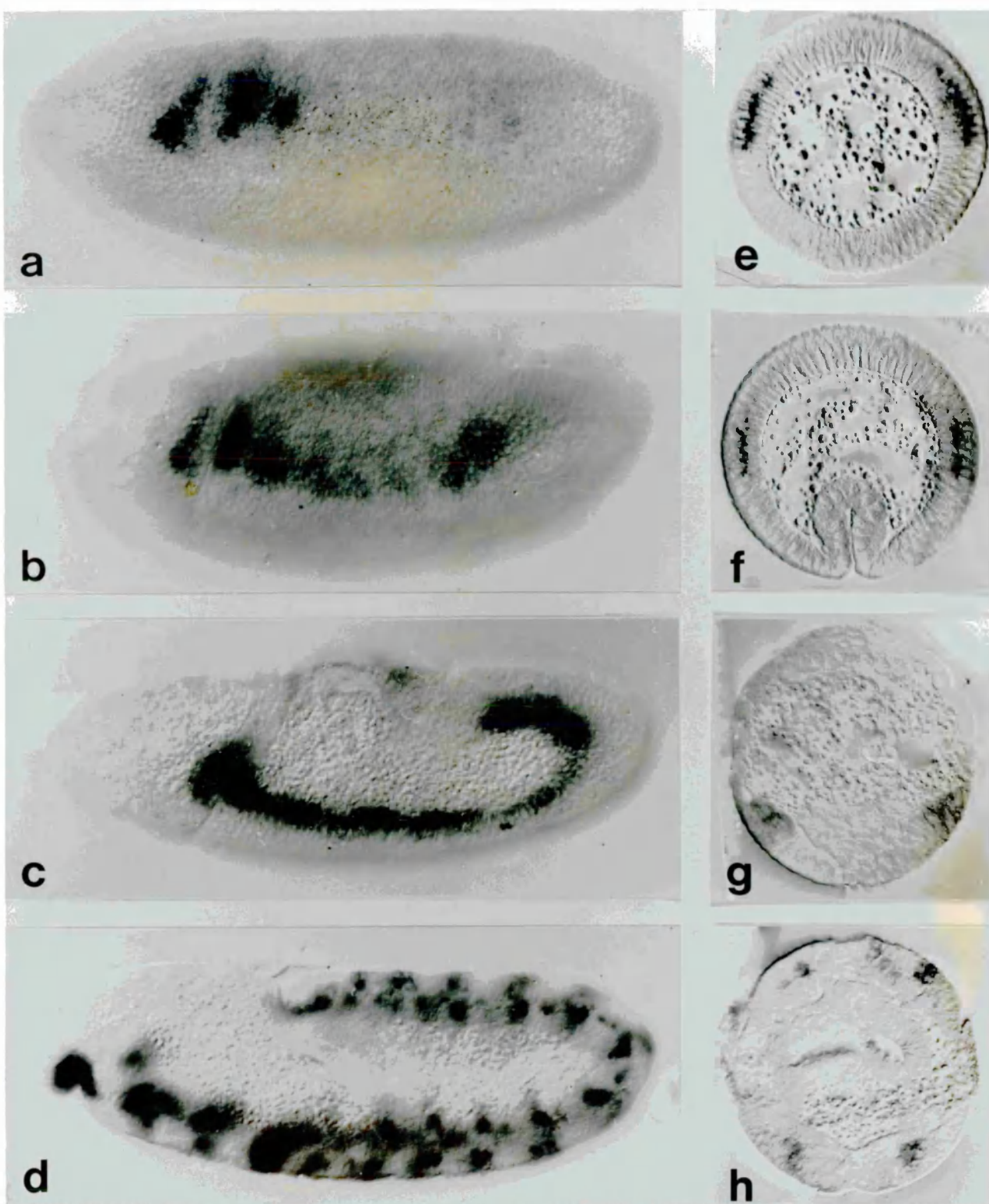
**Figure 3.10. Expression pattern of late *pnr* on *zen*, *srw*, *1xdpp*, and *tsg* mutant embryos.** *In situ* hybridizations on whole mount embryos. Anterior to the left and dorsal up. From top to bottom: The two longitudinal stripes of late *pnr* fuse at the dorsal midline in *zen* mutant embryos. In *srw* and *1xdpp* mutant embryos, expression is wider and appears in a wave-like fashion. In *tsg* mutant embryos, expression extends as far ventrally as in wild-type.

## Effects of zygotic ventralizing mutants on the expression of genes in the neuroectoderm

Since no genes expressed specifically in the ventral ectoderm from the subtractive library screen were identified, we chose a previously identified gene, mesoderm specific homeobox (*msh*) (Gehring, 1987) as a marker for the dorsal part of the ventral ectoderm. At cellular blastoderm, *msh* expression appears initially as three consecutive patches running laterally along each side of the embryo. The two anteriormost patches extend further dorsally and flank the region that will become the cephalic furrow. By stage 6 additional patches arise and a total of eight patches can be seen running anterior to posterior in a lateral stripe along the procephalic and ventral neurogenic region. Cross-sections at stages 5 and 6 reveal *msh* expression to be ventrally adjacent to the *pnr* region. Cell counts show that there is no overlap between the two, but one cannot be absolutely certain due to the gradient expression of *pnr*. Although the expression of the two genes may overlap by one or two cells during these stages, by stage 8 when  $\partial_{14}11$  undergoes its first postblastoderm division (Hartenstein and Campos Ortega, 1985), and the cells of the ventral neurogenic region enlarge, *msh* is clearly restricted to the dorsal third of the neurogenic ectoderm (see figure 3.11). This region corresponds to approximately half of  $\partial_{14}N$ . Furthermore, by stage 7, *msh* expression appears to narrow, most likely due to cell intercalation caused by gastrulation movements, and initially appears as a continuous stripe in the ventral-lateral region of the embryo. By stage 8 however, the stripe of *msh* expression appears more metameric.

In *zen*, *srw*, and *tsg* mutant embryos, *msh* expression is unaffected and appears identical to wild-type. In *dpp* mutant embryos, *msh* expands all the way up to the dorsal midline. However, the most ventral boundary of *msh* is not shifted. Therefore, it appears that instead of the entire neurogenic region expanding, only the part where *msh* is expressed is what extends dorsally. *msh* also expands all the way to the dorsal midline in *tld* and *scw* mutant embryos. In *scw* embryos, staining is more faint and disappears earlier than in embryos mutant for *tld* or *dpp*. Therefore, the region that corresponds to  $\partial_{11}$  in these mutant embryos seems to allow for the expression of genes that are normally expressed only in the ventral neurogenic region. In *sog* mutant embryos, *msh* expression initially appears normal, but is significantly reduced in the trunk region by stage 7. By stage 8, expression in the trunk is no longer present. However, the two anterior patches which extend further dorsally, are unaffected.





**Figure 3.11. *In situ* hybridization pattern of the *msh* gene on wild-type embryos at different stages of development.** Anterior to the left and dorsal up in the case of whole mounts. (A) lateral view of a stage 5 (blastoderm) embryo. Three lateral patches surround the region that will become the cephalic furrow. (E) cross-section of an embryo at the same stage as in A. Staining of 8-9 cells present just below the *pnr* region. (B) lateral view of a stage 7 embryo. Five more patches appear posterior to the first three. (F) cross-section of an embryo at the same stage as in B. Expression appears to extend further ventrally. This is most likely due to an expansion of the cells due to the invagination of the mesoderm on the ventral side. (C) patches have fused and expression extends further ventrally due to cell intercalation caused by germ band extension. (G) cross-section of an embryo at the same stage as in C. Expression in the enlarged cells of the neurogenic region. (D) lateral view of a stage 10 (extended germ band) embryo. *de novo* expression has now appeared. (H) cross-section of an embryo at the same stage as D. Expression appears as a series of spots in the neurogenic region.





dpp



scw



sog



wt



tld



zen

**Figure 3.12. Pattern of *msh* expression in zygotic ventralizing mutants.** *In situ* hybridization pattern of *msh* on whole mount wild-type and mutant embryos. Anterior to the left and dorsal up except for the *tld* embryo which is slightly tilted to show more of the ventral side. All embryos are at stage 8 except for the *sog* embryo which is slightly older. In wild-type embryos, *msh* expression appears as a lateral line on each side of the embryo in the dorsal third of the neurogenic region (014N). Expression in the region just anterior to the cephalic furrow extends further dorsally into 01411. In *dpp*, *tld* and *scw* embryos, *msh* expression extends all the way to the dorsal midline. In *zen*, *srw* and *tsg* embryos (latter two not shown), expression is the same as in wild-type. In *sog* embryos, the lateral stripe fades earlier than in wild-type. In the *sog* mutant embryo shown, *msh* expression is almost completely gone except for the spot anterior to the cephalic furrow.



## Effects of the *twist snail* double mutation on genes expressed in the neuroectoderm

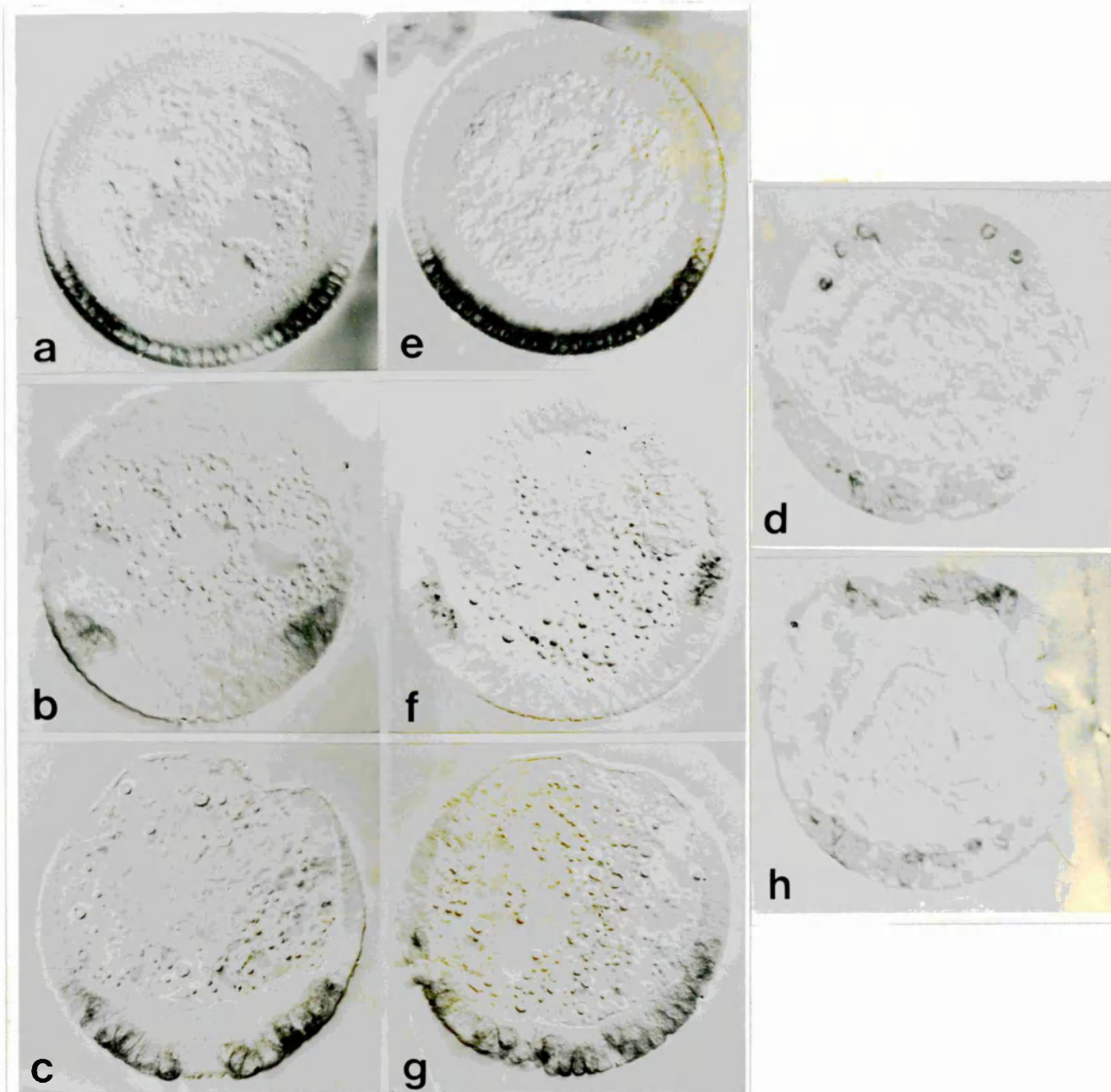
Since none of the zygotic ventralizing mutants affected the ventral boundary of *msh* expression, we decided to investigate whether mutations in more ventral regions would have any influence on this boundary. As suggested previously by Rao et al., (1991), genes expressed in the ventral neurogenic region are shifted to a more ventral position in *twi sna* mutant embryos.

*msh* expression, as seen in figure 3.13 remains in the same position in *twi sna* embryos relative to wild-type. Comparisons of cross-sections between mutant and wild-type embryos stained for *msh* reveals no apparent shift of *msh* even as early as cellular blastoderm. Thus, it appears that *msh* expression is also unaffected by genes involved in mesoderm specification.

The next step was to investigate whether the same holds true for genes expressed in regions ventral to *msh*. We decided to use *rho* as a marker for the ventral part of the neurogenic region. In *twi* and *sna* single mutants *rho* extends into the mesodermal anlage where it is normally suppressed by *sna* in wild-type (Kosman et al., 1991; Leptin, unpublished data). In wild-type, *rho* is expressed as early as syncytial blastoderm as two wide stripes in the ventral half of  $\partial_{14}N$  (see figure 3.13). At the onset of cellularization, a dorsal longitudinal stripe lying in the region of the presumptive amnioserosa also appears. In *twi sna* mutants *rho* expression extends into the region that would normally be occupied by the presumptive mesoderm in wild-type by expanding all the way to the ventral midline. However, the dorsal boundary of *rho* is not shifted to a more ventral position. Thus, within  $\partial_{14}N$  the region between the dorsal boundary of *msh*, and the dorsal boundary of *rho* are not affected by mutations in mesodermal genes. It is important to note however, that a gap consisting of 3-4 cells between *msh* and *rho* expression exists, and no marker that is expressed in this region during this early stage is presently known. Therefore, we could not directly test the effects of mutations in the mesoderm on this region. However, one would also expect genes expressed in this region to be unaffected, since the dorsal boundary of *rho* is not changed in the double mutants.

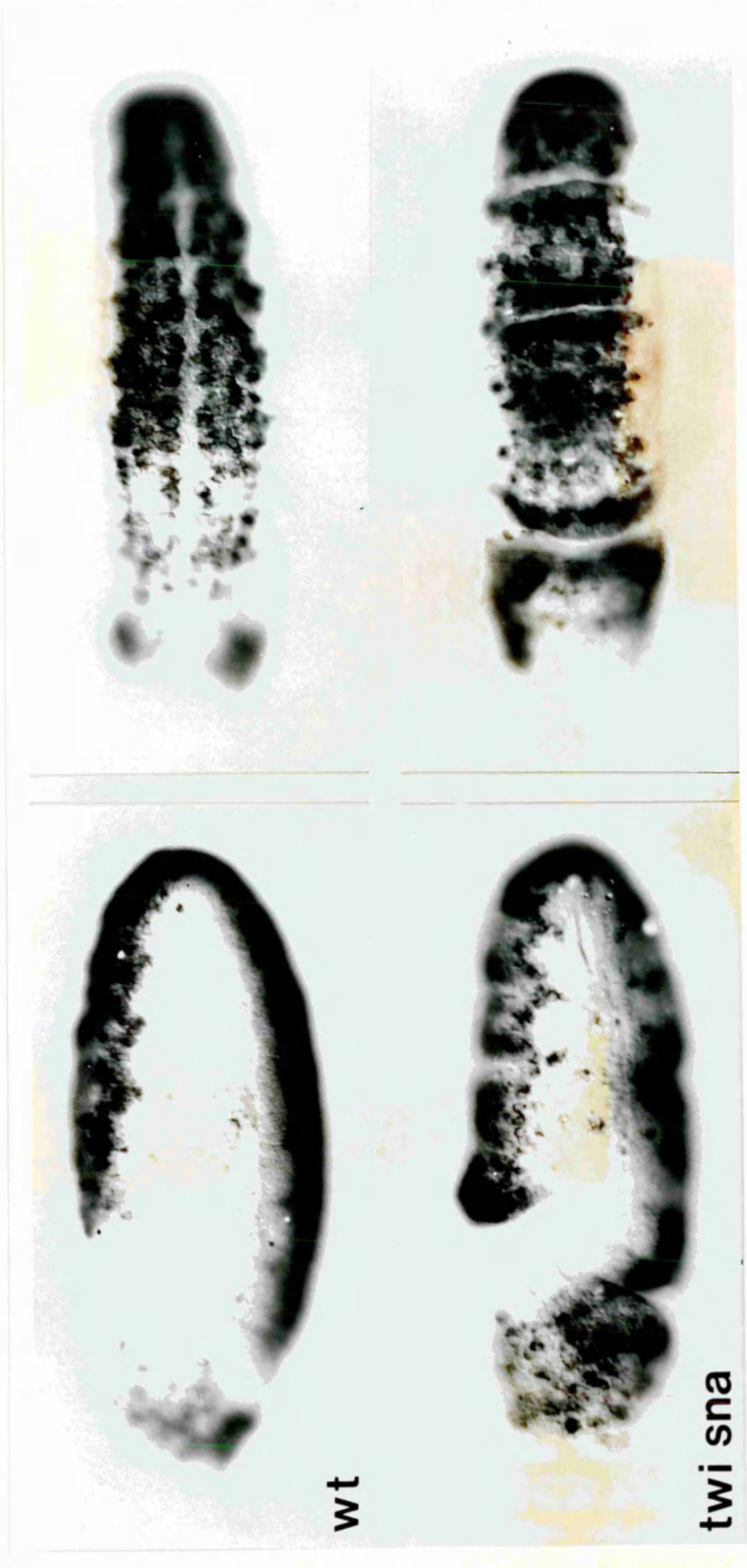
Another possibility was that the shift occurred in later stages. Therefore *sna* was chosen as a marker for neuroblasts. *sna* marks all three subdivisions of the ventral neuroectoderm. However, even after using a neuroblast marker analyzed at the same stages as in the Rao et al (1991) experiment, still no shift in expression pattern was detected. Rao et al. reported a ventral shift in the neuroectoderm in *twi sna*

double mutants because the width of neuronal marker expression in mutant embryos was identical to that of wild-type. Since the mesoderm cells do not invaginate, and remain interspersed among the neuroectodermal cells in these mutants, then the expression of neuronal markers would be expected to be wider if no ventral shift had occurred. However, Rao *et al.* compared stage 5 wild-type embryos to stage 7 mutant embryos. This type of comparison is not valid since cell intercalation has already occurred due to germ band extension in the mutant embryos. Cells along the dorsal-ventral axis are much more compact at stage 7 than at stage 5. Furthermore, simply analyzing the ventral surface of whole mount embryos is not sufficient to judge distances accurately with regard to gene expression patterns due to the curvature of the embryo. One simply must look at cross-sections as well. In early stages, it is clear that there is no shift of the neurogenic region in *twi sna* mutant embryos. By fully extended germ band however, *sna* expression in the neurogenic region no longer appears wider than in wild-type. Nevertheless, the germ band of *twi sna* mutant embryos is significantly longer than that of wild-type embryos and contains many deep folds. Therefore, the entire surface area of this region in *twi sna* mutant embryos is larger than in wild-type. Although the neuroblasts do not appear wider, the total number of cells along the ventral side in *twi sna* mutant embryos is greater. Perhaps it is easier to accommodate these extra cells by expanding in an anterior to posterior direction rather than in a circumferential direction.



**Figure 3.13. Expression patterns of *msh*, *rho* and *sna* on *twi sna* double mutants.** 10  $\mu$ m embryo cross-sections. Right column : wt embryos; left column: mutant embryos. A,E: *rho* expression on wt and *twi sna* double mutant blastoderm embryos respectively. Cellularization is not yet complete. The dorsal boundary of *rho* does not change in the mutant. B,F: *msh* expression in wt and *twi sna* double mutant embryos (stage 8). Cells are more compact and irregular in the mutant. The distance between the two patches of *msh* staining is also greater in the mutant than in wt. This is due to the failure of cells in the mesodermal region to invaginate. Thus, they remain in the ectoderm and cause the cells to become more compact and irregular in shape. C,G: *sna* expression in wt and *twi sna* double mutant embryos (late stage 8, mesoderm has flattened). Expression is wider in the mutant than in wt and irregularities in cell shape are quite distinct. D,H: *sna* neuroblast expression in wt and *twi sna* double mutant embryos (stage 10). Irregularities in cell shape are now very prominent in the mutant embryo and makes cell counts very difficult at this stage (see text). *rho in situ* (Leptin, unpublished).





**Figure 3.14. *sna* neuroblast expression in wild-type and *twi sna* double mutant embryos.** *In situ* hybridization pattern of *sna* on whole mount wild-type and mutant embryos. Lateral and ventral views of wild-type and mutant embryos are shown at the fully extended germ band stage. *sna* expression can be detected in the three rows of neuroblasts that delaminate from the ventral ectoderm. In wild-type embryos, the three rows of neuroblasts are arranged in a very orderly fashion. In *twi sna* embryos cells are more compact and irregular in shape. The distance between the outermost rows of neuroblasts in wild-type and mutant appears the same. However, the mutant embryos contain many deep folds along the length of the germ band. The germ band of *twi sna* embryos is also longer than that of wild-type. (The embryo shown here was simply reduced in size to fit the picture, but is actually larger than the wild-type embryo shown above it).



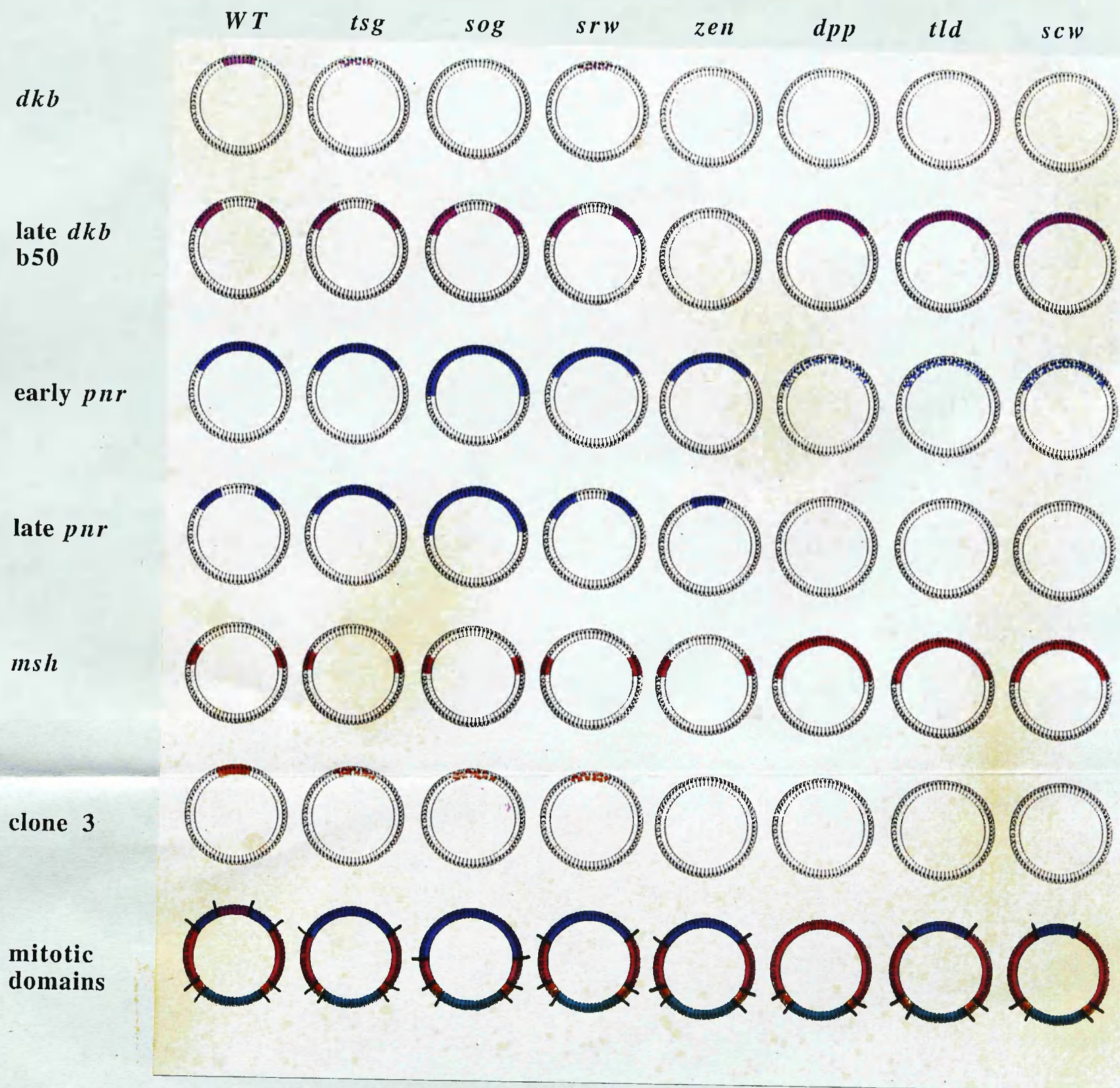


Figure 3.15. Summary of expression patterns of all markers in the zygotic ventralizing mutants as projected onto the blastoderm. Marker names are listed in the left hand column and the genotypes listed in the top row. The bottom row shows the change in mitotic domain pattern of  $\partial A$  (in pink),  $\partial_{14}11$  (in blue),  $\partial_{14}N$  (in red),  $\partial_{14}M$  (in orange), and  $\partial_{14}10$  (in green) (based on data reported by Arora and Nüsslein-Volhard, 1992). The mitotic pattern of *sog* and *tsg* mutants have been extrapolated based on the change in expression patterns of the various markers used. Stippling indicates that the expression is weaker compared to wild-type.

**CHAPTER 4**  
**DISCUSSION**



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## DISCUSSION

### Subtractive library screen

Ideally, it was expected that all dorsal-laterally expressed genes between embryonic stages 5 and 10 would be identified from screening the subtractive library. Thus, a wide variety of genes were expected to have been isolated. Indeed genes were found that were expressed either in specific regions such as the amnioserosa, the tracheal system, or parts of the dorsal epidermis, or over broader regions such as the entire dorsal-lateral part of the embryo. However, despite the fact that almost two dozen novel genes were isolated from the screen, only two previously identified genes were found. In addition, genes expressed specifically within the neurogenic region were not identified. One probable reason is that the screen simply has not reached saturation. The fact that the majority of clones obtained from the dorsal-lateral library screen were only identified once suggests that this is most likely the case. If saturation had been reached, then the majority of clones would be expected to be represented more than once, resulting in a curve resembling a Poisson distribution. In addition, many previously identified genes known to be specifically expressed in the dorsal-lateral regions of the embryo such as *dpp* were not isolated during the screen, even though Southern blot analysis showed that *dpp* was present in the library and significantly enriched after each round of subtraction. Additional rounds of subtraction would expectedly yield a whole new set of dorsal-lateral specific clones. This is what one would expect if the efficiency of subtraction was always high, and that no biases in the system existed. However, this was not the case, as demonstrated by the predominance of the amnioserosa clone b23 which was isolated 41 times out of 296 clones in the second screen.

One mechanism in which a bias might occur is based on the fact that genes are transcribed at different rates with some genes being transcribed tens to thousands of fold more than other genes. Therefore, the cDNAs for certain genes are already present in abundance even before the enrichment process. These genes are consequently enriched further compared to the rare genes. Theoretically, these rare genes should nevertheless be enriched since common genes and genes expressed in higher abundance in the opposite library should be subtracted away. However, the hybridization process is never one hundred percent efficient. One would either have to perform several more rounds of subtraction to enrich for these rare genes, screen several thousand more clones, or perform a screen that combines these two possibilities.

Another factor that must be taken into consideration is the fact that the full expression pattern of each gene was not always revealed using 400bp fragments as probes. This is best exemplified in the case of the b29 clone. Although faint staining was observed in the dorsal epidermis, the full expression pattern was not detected and it was only through sequence analysis that the b29 clone was discovered to be the *tld* gene. Therefore, the clones that did not give specific *in situ* hybridization expression patterns must be reanalyzed using more sensitive detection methods such as the use of RNA probes. If some of these clones actually do give a specific expression pattern, then this may alter the numerical results obtained in the screen. It may also reveal whether some of the clones that gave late expression patterns also have earlier expression patterns. Notably, a number of clones that did not give a specific *in situ* hybridization pattern were isolated a significant number of times. If these clones were simply PCR artifacts, then one would not necessarily expect these clones to be picked up several times. This lends further evidence to the possibility that the 'non-specific' clones are in reality, true dorsal-lateral specific genes that may simply be expressed at lower abundance and were therefore not detected using the procedures used.

If one assumes that the bias in the screen resulted from differences in rates of transcription, then it is not clear why *dpp*, a gene that is expressed over the entire dorsal part of the embryo, and clearly enriched in the dorsal-lateral library after each round of subtraction was not identified. This indicates that perhaps an insufficient number of colonies were screened. However, for practical purposes, one cannot screen tens of thousands of colonies in order to ensure that each dorsal-lateral specific gene will be represented at least once. A more efficient strategy would be to perform several more rounds of subtraction until the majority of clones are dorsal-lateral specific.

Despite the bias caused by the amnioserosa clone, a fair number of genes expressed in the epidermis and the trachea were also obtained. It is not clear however, why no clones expressed in the ventral neurogenic region were isolated. Perhaps they simply were not picked up by chance, or only a few exist. However, it is important to keep in mind that due to the inherent biases in the screen, it is not clear what one would expect to find.

Another possibility is that certain genes are simply more amenable to PCR than others, and are therefore more enriched. One way to determine whether the bias is due to differential transcription of the genes or whether it is due to preferential PCR of specific genes would be to take several clones that were highly represented, and several clones that were represented only once and hybridize them to the original pool



of unsubtracted cDNAs to determine whether the differences in representation already existed before enrichment. One would also have to perform a Northern blot to determine the abundance of transcripts even before PCR. Another strategy would be to perform an additional round of subtraction and use the highly abundant clones as a driver so as to subtract them away from the dorsal-lateral pool. Alternatively, it is also possible that certain cDNAs simply contain very few, or do not contain any Alu I or Rsa I restriction sites. Thus, they would not have been selected since fragments between 200 and 2000 bp were chosen to create the library.

Moreover, this type of screen would only identify genes that are either present in higher abundance in one population of mRNAs versus another, or completely present or absent in one population. Since the majority of genes involved in differentiation of the dorsal and lateral regions are also expected to be expressed in these regions, then a substantial number of genes involved in dorsal-lateral patterning can be identified using this screening strategy. However, one would not identify any ubiquitously expressed genes that may have important functions in dorsal-lateral patterning. One example is the *scw* gene, which is specifically required for proper dorsal differentiation but is ubiquitously expressed throughout the embryo (Arora, 1994).

## PATTERNING ALONG THE DORSAL-VENTRAL AXIS

### What specifies the amnioserosa?

Since the amnioserosa is missing in all of the zygotic ventralizing mutants, it appears that all of the genes from this group directly or indirectly play a role in the specification of this tissue. The current model postulates that the highest levels of *dpp* activity are essential for the formation of the amnioserosa and that all other members of the *dpp* group play a role in increasing the activity of *dpp* within the dorsal side of the embryo. However, it is still unclear as to how these genes carry out this function, whether any of them interact directly with one another, and whether parallel pathways also exist.

The requirement for *tld* and *scw* is not absolute however, since increased levels of *dpp* activity through the introduction of extra copies of the gene can rescue the *tld* and *scw* mutant phenotype (Ferguson and Anderson, 1992a). Thus, the specification of the amnioserosa,  $\partial_{1419}$  and  $\partial_{1411}$ , seem to depend only on the level of *dpp* activity. Analysis of the genetic interactions between *dpp* and other members of the *dpp* group suggests that *zen* acts downstream of *dpp*, since increased doses of the *dpp* gene do not rescue the *zen* mutant phenotype (Ferguson and Anderson, 1992a). Indeed subsequent experiments proved this to be the case. When *dpp* RNA was injected into

lateralized embryos void of *twi*, *dpp*, *tld* or *zen* expression, high quantities of *dpp* RNA were able to drive amnioserosa differentiation. In addition, *zen* transcripts were detected at the site of injection, confirming the hypothesis that *zen* acts downstream of *dpp* (Ferguson and Anderson, 1992b). In a similar experiment, an increase in the dosage of the *dpp* gene in *dl* mutant embryos resulted in the differentiation of amnioserosa cells, only in the regions where *zen* was expressed (Ray, 1993). Hence, these data demonstrate that both *dpp* and *zen* are essential for promoting the amnioserosa fate, but either one alone is not sufficient. High levels of *dpp* function to specify the amnioserosa cell fate and activate *zen*, while *zen* promotes the proper differentiation of amnioserosa cells.

### **What genes function downstream of *zen*?**

The absence of expression of the dorsal-lateral clones *dkb*, 3 and 26 in *zen* mutant embryos suggest that they function downstream of *zen*. However, the presence of the *dkb* collar and posterior spot in *zen* mutants implies that the expression of *dkb* is not exclusively initiated by *zen*. In addition, the *dkb* dorsal stripe is quite variable and sometimes appears wider than the refined *zen* expression. Another gene with a similar expression pattern has recently been cloned. This gene is *u-shaped* (*ush*) and is expressed in an identical manner as the *dkb* dorsal stripe (Ray, 1993). The *ush* expression pattern is also wider than the refined *zen* expression, thus suggesting that this gene is not exclusively initiated by *zen* as well. Thus, *ush* and *dkb* may define an additional threshold at which the *dpp* gradient is interpreted.

### **Possible role of early *zen* expression in specifying the dorsal epidermis**

In *zen* mutant embryos early *pnr* expression is the same as in wild-type. In older mutant embryos the ventral boundary of late *pnr* expression is shifted dorsally so that the two longitudinal stripes of *pnr* fuse at the dorsal midline. In wild-type embryos, *pnr* expression is repressed from the amnioserosa at this stage. Since the amnioserosa is missing in *zen* embryos, one could interpret this dorsalmost expression as a derepression of *pnr*, and a consequent dorsal expansion of  $\partial_{14}11$ . A simple expansion of the dorsal border of  $\partial_{14}11$  into  $\partial A$  is unlikely however, since the ventral border of *pnr* is also shifted. Since the ventral boundary of *pnr* is shifted dorsally, one would expect that at least the dorsal boundary of the *msh* region would also shift dorsally. However, the *msh* region does not expand into the region previously occupied by *pnr*. If *pnr*, a marker for  $\partial_{14}11$ , and *msh*, a marker for the dorsal part of  $\partial_{14}N$  do not occupy this region, then it is not clear what fate this region acquires in *zen* mutant embryos. Based on this observation one would assume that this region acquires a completely different type of fate not normally found in wild-type. Nevertheless, pattern elements such as fine dorsal hairs are still present in *zen* mutant cuticles. Thus,

it appears that this region still retains at least some characteristics of the dorsal epidermis despite the fact that it no longer expresses *pnr*.

If one considered the early and late expression patterns of *pnr* as separate entities, then one could envisage the following scenario as a possible explanation: early *zen* expression is required to maintain late *pnr* expression in  $\partial_{1411}$  in conjunction with *dpp*, *tld*, and *scw*, while refined *zen* expression acts to suppress late *pnr* expression in the amnioserosa region. Evidence that early *zen* expression can still influence the specification of cell fate through the expression of certain genes in later stages is shown by the expression of amnioserosa specific markers such as clone 3 and *dkb* in *srw* and *tsg* embryos. Although this only demonstrates the late action of early *zen* expression in the amnioserosa region, one can nevertheless imagine that the early expression of *zen* in  $\partial_{1419}$  and  $\partial_{1411}$  may also have a late effect on gene expression in these domains. Another possibility is that the activity of refined *zen* expression has a long range effect on the expression of certain genes in adjacent regions  $\partial_{1419}$  and  $\partial_{1411}$ . This long range effect would most likely have to be carried out by genes that encode secreted proteins downstream of *zen*.

Similarly, the same hypothesis for early *zen* function can be applied toward the late pattern of *dkb*. Unlike the late *pnr* expression, where two longitudinal stripes occupy the dorsal part of the embryo, *dkb* expression is absent in *zen* mutant embryos. One explanation may be due to the fact that *dkb* is expressed further ventrally than *pnr*. It is possible that only the  $\partial_{1419}$  expression of *pnr* is what extends into the refined *zen* domain (amnioserosa region) and the remaining expression that lies in  $\partial_{1411}$  is absent because there is no early *zen* to maintain it. Some evidence exists to support this. In *zen* mutants, the two late dorsal stripes of the *dpp* pattern which mark  $\partial_{1419}$ , fuse at the dorsal midline. However, the expression of another marker for  $\partial_{1411}$  does not shift to a more dorsal position (Ray, 1993). Moreover, when the *string* gene was used as a marker for mitotic domains in *zen* mutant embryos, expression in  $\partial_{1411}$  did not shift dorsally (this work, data not shown). Thus, this evidence suggests that only  $\partial_{1419}$  extends into the refined *zen* domain.

As in the case of *zen* embryos, late *pnr* expression extends all the way to the dorsal midline in *tsg* mutant embryos. However, the ventral boundary of late *pnr* expression in *tsg* embryos remains the same as in wild-type. This region is normally occupied by the amnioserosa which no longer expresses *pnr* at this stage. In *tsg* mutants, the amnioserosa is essentially missing (except for the few cells that still express amnioserosa specific markers). Thus, it appears that the amnioserosa acquires characteristics of the region just ventral to it in *tsg* mutants as defined by *pnr*



expression. Hence, one could define the amnioserosa region in *tsg* mutants as being transformed into the dorsal epidermis. In *tsg* embryos, *zen* expression does not refine. This pattern can be considered as the early *zen* pattern, which is required for maintaining *pnr*. Thus, *pnr* is not suppressed by refined *zen* expression, and therefore extends to the dorsal midline. In *srw* embryos, early *zen* expression rapidly degrades. However, the presence of amnioserosa cells in later stages in these embryos demonstrates that even a short phase of *zen* expression is not only sufficient to activate the amnioserosa differentiation pathway, but also sufficient to maintain *pnr* in  $\partial_{14}11$ .

In embryos carrying one copy of the *dpp* gene, the amnioserosa is missing (Wharton et al., 1993), but the rest of the dorsal region remains intact. The spatial expression of *pnr* also appears to be slightly narrower. However, this apparent shift of *pnr* expression to a more dorsal position can be attributed to the failure of cells in the amnioserosa region to flatten into a thin sheet in embryos carrying only one copy of the *dpp* gene. Thus, germ band extension is inhibited and as a consequence, cell intercalation does not occur and *pnr* remains in a more dorsal position rather than being pushed to a more lateral position. Thus, the effects of only one dose of the *dpp* gene is not equal to a null mutation of *zen* because of the effects of early *zen* function. Similarly, the effects of only one dose of the *dpp* gene is not equal to a null mutation of *tsg*, because *tsg* probably acts along a separate pathway since extra copies of *dpp* do not rescue the *tsg* mutant phenotype (Ferguson and Anderson, 1992a). Out of all the zygotic ventralizing mutants *srw* most closely resembles the 1x *dpp* mutant phenotype with regard to *pnr* expression. This correlates well with the proposed function for *srw*, which is to increase the level of *dpp* activity. Deleting the *srw* function would lower *dpp* activity in a similar manner as removing one copy of *dpp*.

### **Possible interactions between the *dpp*, *tld* and *scw* gene products**

The identical effects of the *dpp*, *tld* and *scw* mutations on the early expression patterns of all the markers used in this study suggest that either all three genes have similar functions or that all three act along a common pathway. Furthermore, the structures of the *dpp*, *tld* and *scw* gene products suggests that there may even be possible direct interactions among the three. The *dpp* and *scw* gene products belong to the TGF- $\beta$  superfamily of secreted growth factors (Padgett et al., 1987; Arora, 1994)), while the *tld* gene product shows high homology in sequence and structure to *bone morphogenetic protein 1* (*BMP-1*; Wozney et al., 1988; Shimell et al., 1991). Both the *tld* and the *BMP-1* protein possess a zinc-binding metalloprotease domain very similar to the astacin protease of the crayfish *Astacus fluviatilis* (Zwilling and Neurath, 1987). Data based on the mutational analysis of the *tld* gene suggest that the *tld* protein may form a complex with the *dpp* protein and that the protease domain is



required for the activation of the *dpp* complex (Finelli et al., 1994). Previous work in vertebrates has shown that the gene products of different TGF- $\beta$  family members are capable of forming heterodimers with one another when expressed within the same cell (Cheifetz et al., 1987). Thus, the *dpp* and *scw* gene products may form heterodimers with one another, while *tld* may act to modify this complex with its protease activity. This is most likely the case in the regulation of the *msh* gene since the expression pattern of *msh* is identical in all three mutants.

### **What specifies the dorsal part of the neuroectoderm (*msh* region)?**

In *dpp*, *tld* and *scw* mutant embryos, the dorsal boundary of *msh* expression extends up to the dorsal midline. The ventral boundary of *msh* expression however, is not affected. This suggests that *dpp*, *tld* and *scw* play a role in repressing *msh* in more dorsal regions of the embryo. In *dpp* mutant embryos, mitotic domains  $\partial A$ ,  $\partial_{14}19$  and  $\partial_{14}11$  are absent, and the region of  $\partial_{14}N$  where *msh* is expressed extends all the way to the dorsal midline. Thus, the change in the *msh* expression pattern correlates with the change in mitotic domain pattern in *dpp* mutant embryos. In *tld* and *scw* mutant embryos, mitotic domains  $\partial A$ ,  $\partial_{14}19$  and parts of mitotic domain  $\partial_{14}11$  are absent. Mitotic domain  $\partial_{14}N$  shifts to a more dorsal position but does not extend all the way to the dorsal midline. Thus, in *tld* and *scw* mutants, the change in the *msh* expression pattern does not correlate with the change in mitotic domain pattern. In addition, *pnr* is still expressed (albeit more faintly) in *tld* and *scw* mutant embryos. Therefore, the subset of  $\partial_{14}11$  cells that remain in these mutants is transformed into a cell population possessing both characteristics of the dorsal part of mitotic domain  $\partial_{14}N$  as defined by *msh* expression, and characteristics of mitotic domain  $\partial_{14}11$  as defined by its mitotic pattern, and the expression of *pnr*. In other words, this subset of mitotic domain  $\partial_{14}11$  in *tld* and *scw* mutants appears to have acquired 'mixed fates'. Notably, in the larval cuticle, dorsal and dorsal-lateral pattern elements can still be detected in these mutants. One possible explanation is that late regulation by other patterning genes corrects this situation and rescues cells in  $\partial_{14}11$  from completely acquiring a more ventral fate.

Nevertheless, the questions still remains as to how this apparent fate mixing occurs during earlier stages. If we consider the *dpp* gradient model in which high levels of *dpp* activity are necessary to specify amnioserosa, intermediate levels to specify dorsal epidermis, and low levels or the absence of *dpp* activity to specify more ventral fates, then a decrease in *dpp* activity in *tld* and *scw* mutants would remove the amnioserosa and some parts of  $\partial_{14}11$ . Therefore, based on this model, *tld* and *scw* mutants can be considered as possessing only intermediate to low levels of *dpp* activity. This leads to a paradoxical situation in which these mutants contain intermediate levels of *dpp*

activity and yet express markers such as *msh* that are normally expressed only in regions with low levels of *dpp* activity. Given these data, it appears that the mechanism for *msh* repression is not dependent on the level of *dpp* activity.

To resolve this matter one would have to envisage a model which involves two separate pathways. The first pathway would involve the current model where specification of the amnioserosa, mitotic domains  $\partial_{1419}$  and  $\partial_{1411}$  are dependent on the levels of *dpp* activity, while the second pathway would involve the activation of a *msh* repressor that is independent of the level of *dpp* activity. The simplest model would be to assume that all three genes, *dpp*, *tld*, and *scw*, are essential for the activation of the *msh* repressor. The absence of either one of the three genes would lead to the complete collapse of the repressor activation complex. Furthermore, the requirement for all three genes suggests that there may even be direct interactions between the *dpp*, *tld* and *scw* gene products. This model would involve two different types of receptors for *dpp*. Both receptors would be expressed in the dorsal epidermis ( $\partial_{1411}$ ) except that one would have a much higher affinity for the *dpp/dpp* homodimer while one would have a higher affinity for the *dpp/scw* heterodimer. Binding of the *dpp/dpp* homodimer to its receptor would activate the signalling pathway for dorsal epidermis differentiation, while binding of the *dpp/scw* heterodimer would activate the signalling pathway for repression of *msh*, and possibly other genes expressed in  $\partial_{14N}$ . The *tld* protein would function to modify the *dpp/dpp* homodimer or *dpp/scw* heterodimer to facilitate binding to the appropriate receptor. Alternatively, the *dpp* protein may form a heterodimer with another TGF- $\beta$  molecule other than the *scw* protein to promote the activation of the *msh* repressor, while the *scw* protein would somehow be involved in a separate signalling pathway that produces the TGF- $\beta$  molecule, or vice versa.

Another model would involve the action of some maternal gene to activate the *msh* repressor, as well as the concerted action of *dpp*, *tld* and *scw* for repressor maintenance. This repressor would require the activity of all three genes in order to be active at a high enough level to repress *msh*. This model would require however that *msh* expression be very sensitive to the threshold level of the repressor. Even a small decrease in repressor activity would no longer be sufficient to repress *msh*.

### **What specifies the ventral boundary of *msh* expression?**

In *twi sna* mutant embryos, the ventral boundary of *msh* expression remains in the same position as in wild-type. In contrast, the ventral boundary of *rho* expression in *twi sna* mutant embryos is shifted to the ventral midline. The dorsal boundary of *rho* expression however, is unaffected. Thus, mutations in genes that affect patterning of

the most ventral regions of the embryo do not appear to affect the expression of genes within the region between the dorsal boundary of *msh* and the dorsal boundary of *rho*. It is unlikely that *rho* specifies the ventral boundary of *msh* since a gap of 3-4 cells exists between *rho* and *msh* expression in the blastoderm. In *sog* mutant embryos, *msh* expression degrades earlier than normal. However, this may simply be a consequence of the action of *sog* on *dpp* expression. In *sog* mutant embryos, an increase in *dpp* transcripts can be detected in the *msh* region (Roth, personal communication). This implies an increase in the *dpp* product and a possible corresponding increase of the *msh* repressor activation complex above the limiting threshold level. A corresponding increase in the *tld* protein might also be expected in *sog* mutant embryos in order to bring the level of the *msh* repressor activation complex above the threshold level. However, it is also entirely possible that a simple increase in *dpp* would provide enough substrate for the low level of *tld* to act upon. Given this model, it is unlikely that *sog* specifies the ventral boundary of *msh*.

It is quite likely however, that another gene ventrally adjacent to *msh* acts to repress *msh* in more ventral regions. Indeed some preliminary evidence to support this model exists. A novel embryonic mutant M68 has been identified that in combination with the *sog* mutation allows for the expression of *dpp* all the way to the mesoderm/ectoderm boundary (Roth, personal communication). Thus, another gene in addition to *sog* appears to act further ventrally to regulate *dpp* activity in a negative manner. A simple way to test whether this gene specifies the *msh* boundary is to analyze the expression pattern of *msh* in M68 mutants. The above model is based on the assumption that *msh* can be activated in more ventral regions. Conversely, the activator of *msh* may only be expressed in the *msh* region and further dorsally but not in ventral regions.

**CHAPTER 5**  
**CONCLUSIONS**



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## CONCLUSIONS

The results obtained from the experiments presented here reveal that the apparent fate shifts described for the zygotic ventralizing mutants as demonstrated by changes in cuticular pattern and mitotic domain pattern are not always mimicked by corresponding shifts in gene expression patterns. Thus, perhaps a more appropriate term to describe the changes in gene expression patterns observed in the zygotic ventralizing mutants would be 'ectopic gene expression', rather than simple fate shifts.

Given the structure of the *dpp*, *tld* and *scw* gene products, and the fact that loss-of function mutations in these genes resulted in identical changes in expression patterns of *all* the markers used in this study reinforces the possibility that all three gene products not only act along a common pathway, but interact directly with one another. Furthermore, parallel pathways rather than a single pathway to pattern the dorsal side is much more likely. This is evidenced by the effect of the *dpp*, *tld* and *scw* mutations on the expression of the *msh* gene, as well as the effect of the *tsg* mutation on all the dorsal epidermis markers used.

The analysis of the effects of the *zen* mutation on genes expressed in the dorsal epidermis suggests a possible role for *zen* in patterning this part of the embryo. It cannot be determined however, whether the influence of *zen* on the expression of genes in the dorsal epidermis is due to the early expression of *zen* or a long range effect of the refined *zen* expression. Nevertheless, the presence of some amnioserosa cells in *srw* embryos suggests that even a short pulse of *zen* expression is sufficient to specify a few cells to acquire the amnioserosa fate.

Since none of the mutations analyzed affected the ventral boundary of *msh* expression, it appears that none of the zygotic ventralizing genes or any of the ventral patterning genes are required to set up the early boundary between dorsal and ventral ectoderm. Thus, it appears that the lateral region of the embryo may be patterned independently of zygotic dorsal and ventral patterning genes. Perhaps the ventral boundary of *msh* is directly specified by the *dorsal* gradient. Analysis of *msh* expression in *dl<sup>-</sup>* mutants would answer this question. The analysis of *msh* expression in *dpp twi sna* triple mutants (as well as additional markers from this region that could potentially be obtained from a rescreen of the subtractive library) may also provide more insights into how the early boundary between the dorsal and ventral ectoderm is established.

In summary, it can be concluded that the zygotic ventralizing genes function to set up boundaries within the dorsal half of the trunk region of the embryo and determine cell fates within these boundaries. Specific qualities of any one region can be affected differently by different genes. In addition, although *dpp* clearly plays a major role in dorsal patterning, there appears to be no linear hierarchical order among the zygotic ventralizing genes. Instead, they appear to function through a series of parallel pathways that act simultaneously to pattern the dorsal side of the embryo.

CHAPTER 6  
BIBLIOGRAPHY

## BIBLIOGRAPHY

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**CHAPTER 7**  
**APPENDICES**

**APPENDIX A**  
(numbers regarding the dorsal-lateral library screen)

**FIRST SCREEN (6TH ROUND OF SUBTRACTION)**

**750** COLONIES SCREENED  
**60** WERE DORSAL-LATERAL SPECIFIC  
**54** HAD PICKABLE COLONIES  
**29** DIFFERENT CLONES AFTER DOT BLOT  
CROSS-HYBRIDIZATION ANALYSIS  
**12** OF THE 29 GAVE SPECIFIC EXPRESSION  
PATTERNS AFTER *IN SITU* HYBRIDIZATION  
ANALYSIS  
**1 7** GAVE NO SPECIFIC EXPRESSION PATTERN

**SECOND SCREEN (7TH ROUND OF SUBTRACTION)**

**3000** COLONIES SCREENED  
**296** WERE DORSAL-LATERAL SPECIFIC  
(ALL PICKED)  
**152** HYBRIDIZED TO THE 12 DORSAL-LATERAL  
SPECIFIC CLONES FROM THE FIRST SCREEN  
**20** HYBRIDIZED TO THE 17 NON-SPECIFIC CLONES  
FROM THE FIRST SCREEN  
**1 2 4** CLONES REMAINED  
**1 8** DIFFERENT CLONES AFTER DOT BLOT  
CROSS-HYBRIDIZATION ANALYSIS  
**1 2** OUT OF THE 18 GAVE SPECIFIC EXPRESSION  
PATTERNS AFTER *IN SITU* HYBRIDIZATION  
ANALYSIS  
**6** GAVE EXPRESSION PATTERNS IDENTICAL TO  
SOME OF THE CLONES FROM THE FIRST SCREEN  
**6** GAVE NEW EXPRESSION PATTERNS



## APPENDIX B

(DNA sequences of a number of clones obtained from  
the dorsal-lateral library)

### DL 3

Dl3sk.Seq Length: 159

```
1 acagataagc attacagaga atactgagaa taagatcttc aagtatcaga
51 gagaagtgca agtgggtcaac ggatntcaat catttaacca gatatcgagc
101 atctacacaa gttcccatct taccggaagt ggaatacttt ctgtggtaaa
151 aactacgg
```

### DL 15

Dl15.Seq Length: 107

```
1 acacagcgac aagttttccg cgcacacgga aagtcagcaa gttaatacac
51 cgaaatatat agtttcaaat ccgactaacg tttcaaatcg aaattacctg
101 tgagtgc
```

### DL 19

Dl19.Seq Length: 180

```
1 ggatgtggac gacaagaaga ctaagccact tccggtcgct tctactctcc
51 tcatcacatc cacaccacaa ctcatgcaca tgcacacgca cacacaagca
101 cactgaaaga aattatcaat gaaatcaaat cgaacaatt tcgaaacaaa
151 acaacacaca gattcgcgtc ttagtcactt
```

### DL 20

Dl20sk.Seq Length: 66

```
1 atttcngacc atccgaatcg ccgatcgagc atttcctccg gctgtctcac
51 cgcctccttc gttggc
```

## DL 26

Dl26ks.Seq Length: 70

```
1  atacnaactg aatcctattg tcgcgtcctt ggacgtcggg atggttctgg
51 gttataactt ctttgcgtgg
```

## DL 31

Dl31ks.Seq Length: 208

```
1  ctgaagactc ggattttcgc tggttttgca actgcaacaa aggcggcttc
51  tgttggccat tggaaactgga ttggctggac ttgaggcnan ggttgctcga
101 acagcggctg cttgcggatg gtcagtggct tcgctttgat gggtaaattg
151 tcgttgatgt taagggattg cgtncaaagg tggcctttgc gtctnattag
201 cagtcgat
```

## DL 33

Dl33ks.Seq Length: 74

```
1  gaagaaggcc gtcgttgca agattatggt caccatcatc aagttcatct
51  cgctgaaggc tctggcatct cctt
```

## DL 36

dl36KS.seq Length: 182

```
1  ttcggactag gagcaccctg ctctggcca tttttgtgca gcaaacgcta
51  gcacagcgaa ccccgaccat atnctacatc acgcaggagc agatcaagga
101 tatcggcggc acagtggagt tcgatngctc cgtccagtat gccaaagagt
151 acaacgtgct gttcctgaag acggacagcg at
```

## DL 37

Dl37ks.Seq Length: 78

```
1  ctaaaagtgt tttcaaatg aaattcttga ttttctgtct gattgctctg
51  tttgcatag cctcggcncg tncccagt
```

**DL 41**

Dl41ks.Seq Length: 90

1 gtgacgagca ggaggacacc agacggagag ttcaggtcct tgttgctcta  
51 tggtcggaaa cgcagtagcg tcaacaccac agtagtacta

**B50**

Seqb50.Sk Length: 143

1 cagcccggcc ggcaagtgtg tccgtatata cgtagggccca tctctttcgc  
51 tcacccccct tttcgaaggg aagtcagtgc gtgtcgtaat tggcacctct  
101 cattccgcag aacgcggggc acgaacgtag cccgtagcat agc

**B93**

Seqb93.Sk Length: 201

1 gcaacgagac accgatgata gagaaatccg ctttgccgga ggacagcag  
51 gagcacgccg cggagnnnnn gggcagtggg cagggcaaga gtcgccgtcg  
101 caaataggaa aagccaaagt tggcnccacg gagccacgga tacagagata  
151 gaggatagcc aaacttgacac tgcacatgca gagcagaccg tgtctgctac  
201 a

**DL 31 aligned with the *crbs* sequence**

54 cggcggcacctgcctgaccattgCGGgtccagtcggagtgcacatgtcgg 103  
||| ||| . ||||| ||| || || | || ||||| ||||| |  
5980 CGGTGGCCTCTGCCTGACCACTGGAGCGGTAC.CGATGTGCAAATGTAGT 6028  
104 c 104  
|  
6029 C 6029

**B93 aligned with the *Delta* sequence**

91 ctcttgccctgc.ccactgcccnnnnctccgCGGcgtgctcctcgtgt 43  
||||| ||| | || || || |:::| | | | | | |  
2453 CTCTTGCCCTCCGCCGCTCCC GGTCCTCCGCCAGACGCTCCCTTGGCGGA 2404  
42 cct.....cgggcaaagcggatttctcgatc.....atcgggtgtctcgtt 3  
| | |||| | | | | | | | ||||| |||  
2403 CGTGCCCTGCCGGCGAACCGGATGCATGAGCGTGGGATCGGTGTTGAGTT 2354  
2 gc 1  
||  
2353 GC 2352

**DL 12 aligned with the *pdm* sequence**

>gp|M81958|DROPOUB\_1 POU domain protein [Drosophila melanogaster]  
>gp|S64239|S64239\_1 *pdm*-2 gene product [Drosophila]  
Length = 413

**Minus Strand HSPs:**

Score = 134 (68.7 bits), Expect = 2.6e-12, P = 2.3e-12  
Identities = 26/26 (100%), Positives = 26/26 (100%), Frame = -3

Query: 119 QALNMSHMOMEAGGSGSFCGSSISSGE 42  
          QALNMSHMOMEAGGSGSFCGSSISSGE  
Sbjct: 388 QALNMSHMOMEAGGSGSFCGSSISSGE 413

Score = 62 (30.3 bits), Expect = 0.94, P = 0.58  
Identities = 11/11 (100%), Positives = 11/11 (100%), Frame = -1

Query: 154 TPLSSHAFGY P 122  
          TPLSSHAFGY P  
Sbjct: 376 TPLSSHAFGY P 386

**B29 aligned with the *tld* sequence**

LOCUS DRODVP 3374 bp ss-mRNA INV 22-NOV-1991  
DEFINITION D.melanogaster dorsal-ventral patterning (tolloid) mRNA, complete cds.  
ACCESSION M76976  
KEYWORDS dorsal-ventral patterning gene.  
SOURCE Drosophila melanogaster embryonic cDNA to mRNA. . . .

SCORES Init1: 324 Initn: 444 Opt: 453  
97.5% identity in 120 bp overlap

```

                                     10      20      30
Dlb29.                               GGCTGGAGTTCTACNCGGATCGCACTGTCC
                                     |||:|||||
Drodvp CAGTGGTGAATTCCGAGCAGAGTATCCTCAGGCTGGAGTTCTACTCGGATCGCACTGTCC
        2240      2250      2260      2270      2280      2290

        40      50      60      70      80
Dlb29. AGAGAAGTGGATTTGTGCCAAGTTTGTAAATAGATGTGGATGAGTGTTC AAT-AACAATG
        |||:|||||
Drodvp AGAGAAGTGGATTTGTGCCAAGTTTGTAAATAGATGTGGATGAGTGTTC AATGAACAATG
        2300      2310      2320      2330      2340      2350

        90      100      110      120
Dlb29. GTGGCTGTCAGCACCGATGCCGAAACACTTGATC
        |||:|||||
Drodvp GTGGCTGTCAGCACCGATGCCGAAACACTTGGATCCTATCAGTGCAGCTGCCGCAATG
        2360      2370      2380      2390      2400      2410
```