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Allocation of the thoracic imaginal primordia in the *Drosophila* embryo

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SUMMARY

The primordia of the thoracic imaginal discs of the *Drosophila* embryo originate as groups of cells spanning the parasegment boundary. We present evidence that the thoracic imaginal primordia are allocated in response to signals from the *wingless* (*wg*) and *decapen - taplegic* (*dpp*) gene products. Rows of cells that express *wg* intersect rows of cells that express *dpp* to form a ladder-like pattern in the ectoderm of the germ band extended embryo. The imaginal primordia originate as groups of cells which lie near these intersection points. We have used a molecular probe derived from the *Distal-less* (*Dll*) gene to show that this population contains progenitor cells for both the dorsal (i.e. wing) and ventral (i.e. leg) discs. Although we show that *Dll* function is not required for allocation of imaginal cells, activation of an early *Dll* enhancer may serve as a molecular marker for allocation. A group of cells, which includes the imaginal progenitors, activate this enhancer in response to intercellular signals from *wg* and perhaps from *dpp*. We have used a conditional allele of *wg* to show that *wg* function is transiently required for both allocation of the imaginal primordia and for initiation of *Dll* expression in these cells during the brief interval when *wg* and *dpp* form the ladder-like pattern. Allocation of the imaginal primordium and activation of *Dll* expression appear to be parallel responses to a single set of positional cues.

Key words: *Distal-less*, *wingless*, *decapentaplegic*, TGF-β, imaginal disc, pattern formation, limb development, *Drosophila*

INTRODUCTION

The epidermal structures of the adult head, thorax and terminalia of *Drosophila* derive from specialized precursors known as imaginal discs, which originate as clusters of cells in the embryonic ectoderm. Genetic analyses have shown that the disc primordia are first specified as groups of cells (Garcia-Bellido and Merriam, 1969; Garcia-Bellido et al., 1973; Garcia-Bellido et al., 1976; Wieschaus and Gehring, 1976a,b), known as polyclones (Crick and Lawrence, 1975). Somatic mosaic clonal analyses have shown that the imaginal primordia are subdivided into distinct anterior and posterior founder populations. The distinction between anterior and posterior polyclones exists from as early as genetically marked clones can be produced by mitotic recombination (i.e. one cell division after blastoderm; Wieschaus and Gehring, 1976b; Steiner, 1976; Lawrence and Morata, 1977).

The boundary between anterior and posterior compartments is thought to correspond to the parasegment boundary in the embryo. The spatial relationship between the parasegment boundary and the imaginal primordia suggested that the discs are specified in response to positional signals from the *wingless* (*wg*) gene (Simcox et al., 1989; Cohen, 1990). *wg* encodes a member of the Wnt family of intercellular signaling molecules (Rijsewijk et al., 1987). The Wingless protein is secreted and can be detected over a range of 2-3 cells flanking the cells that express *wg* transcript (van den Heuvel et al., 1989; González et al., 1991). Thus the imaginal disc progenitor cells may be allocated in response to an intercellular positional signal transmitted by the secreted Wingless protein.

Morphological studies have documented the locations of the thoracic imaginal discs in the mature *Drosophila* embryo (Auerbach, 1936; Madhavan and Schneiderman, 1977; Bate and Martinez-Arias, 1991). By 10 hours of development the dorsal (wing and haltere) and ventral (leg) disc primordia form clearly recognizable, separate, groups of cells. The presumptive disc cells can be identified on the basis of their shape and histochemical staining properties (Bate and Martinez-Arias, 1991), as well as by expression of a number of genes including *Distal-less* (*Dll*), *vestigial*, and *escargot* (Cohen, 1990; Cohen et al., 1991; Williams et al., 1991; Whiteley et al., 1992; Hartenstein and Jan, 1992). Although the dorsal and ventral disc primordia are well separated when they are first recognizable, analysis of gynandromorphs and somatic mosaics induced by mitotic recombination has suggested that the leg and wing originate in very close proximity in the embryonic ectoderm. Clones of genetically marked cells induced at blastoderm stage were able to contribute to the formation of both the leg and wing discs (Wieschaus and Gehring, 1976b; Steiner, 1976; Lawrence and Morata, 1977). The distance between the centers of the leg and wing primordia was estimated to

be approximately equal to the diameters of the individual primordia (Wieschaus and Gehring, 1976a). These observations suggested that the dorsal and ventral disc primordia are likely to be immediately adjacent or possibly overlapping in the blastoderm stage embryo.

In this report we use molecular markers to trace the origins of the thoracic disc primordia in the early embryo. *Dll* expression labels the nascent leg primordium at approx. 5-5.5 hours of embryogenesis in clusters of cells located near the intersection between rows of cells that express *wg* and those that express *decapentaplegic*. We show that *wg* function is required at this time to initiate *Dll* expression and also for allocation of the thoracic imaginal disc primordia. We have used a molecular lineage tag derived from the *Dll* gene to follow the developmental fate of cells that originate in the ventral thoracic cluster. We observe that some of these cells are displaced dorsally to form the prothoracic, wing and haltere imaginal discs, while cells that remain in the original ventral position form the leg discs. These observations provide molecular evidence to support the proposal that wing and leg imaginal discs may arise from a common primordium in the early embryo.

MATERIALS AND METHODS

Histochemical methods

Affinity purified antibody to vestigial (Vg) protein was kindly provided by Jim Williams and Sean Carroll. The isolation of the *Dll-lacZ* reporter genes, production of antibody to DLL protein and antibody staining protocols are described in Vachon et al. (1992). The *Dll*-304 transgene consists of an early-acting *Dll* enhancer directing expression of *lacZ*. The *Dll*-215 transgene consists of a late-acting *Dll* enhancer directing expression of *lacZ*. Double labellings to visualize β-gal activity followed by RNA in situ hybridization or by antibody staining were done as described previously (Cohen et al., 1991; Vachon et al., 1992). Most fluorescent double labels were photographed by conventional microscopy. The double-labelled image of anti-β-gal and anti-Vg expression in the wing disc (Fig. 7) was taken on a confocal microscope.

Embryo culture

Dll mutant embryos were recovered from a cross of two different mutant alleles, Dll^{SA1} and Dll^{MP}, which lack all *Dll* function. MP is a large deletion removing the entire *Dll* transcription unit. SA1 is a 5.5 kb deletion which removes the exon encoding the amino-terminal 2/3 of the homeodomain. SA1 only affects the *Dll* transcription unit (Cohen et al., 1989; Cohen and Jürgens, 1989). The stocks were marked with *yellow* and balanced with a CyO chromosome carrying a $yellow^+$ duplication (eg: y; Dll^{SA1}/CyO, bw Df(2R)B80 Dp(1;2) y^+). Mutant embryos produced by crossing the two strains are phenotypically identifiable by their *yellow* cuticle. Embryo culture was carried out as described by Simcox et al. (1989). The characteristic markers for the different leg regions are discussed in Table 1 and described in detail by Schubiger (1968).

Strains

The *wg* temperature-sensitive allele IL114 was used for temperature shift experiments (Nüsslein-Volhard et al., 1984). An enhancer detector insertion in the *wg* gene was used in studies to histochemically visualize *wg*-expressing cells (Perrimon et al., 1991). Isolation of the early and late-acting *Dll* enhancer-*lacZ* fusion strains is described in Vachon et al. (1992). The early-acting enhancer is called *Dll*-304. The late-acting enhancer is called *Dll*-215.

RESULTS

Molecular markers for the imaginal primordia

To visualize the development of the imaginal primordia throughout embryogenesis we made use of molecular markers from the *Distal-less* (*Dll*) and *vestigial* genes (Cohen, 1990; Williams et al., 1991). We will first describe the expression of these markers late in embryogenesis when the disc primordia are morphologically recognizable. Double-labelling experiments used a late-acting *Dll* enhancer element to direct expression of β-galactosidase (β-gal) protein in the leg disc primordia and antibody to Vestigial protein (Vg) to label the wing and haltere discs (Fig. 1). The wing and haltere discs invaginate as simple epithelial sacs, which can be visualized as clusters of cells that express Vg protein, in the late embryo (grey, Fig. 1A). The location of the leg disc clusters can be visualized by expression of β-gal protein under control of the *Dll* late enhancer (brown, Fig. 1A). The leg discs also invaginate, but remain closely apposed to the larval ectoderm (Bate and Martinez-Arias, 1991). In addition to the dorsal discs, Vg is expressed in several sets of larval muscles and in the CNS in each segment, and perhaps in adepithelial cells associated with the thoracic discs.

The leg and wing disc progenitor cells can be distinguished on the basis of gene expression and position before they become morphologically distinct from the larval ectoderm. Cells that will give rise to the wing and haltere discs derive from groups of about 30 cells that express Vg protein in the second and third thoracic segments at about 10 hours of embryogenesis (grey, Fig. 1B). When the wing and haltere discs invaginate, they contain approximately 24 and 12-15 Vg-expressing cells respectively, in good agreement with the numbers reported by Bate and Martinez-Arias (1991) based on histochemical staining. It is possible that the initial expression of Vg in clusters of approx. 30 cells may reflect an initially broad domain of expression that resolves as the disc invaginates. At 10 hours the clusters of Vg-expressing cells are separated by 3-5 cells from the leg primordia, which express *Dll*.

Early expression of *Dll* may provide a molecular marker for allocation of thoracic imaginal cells

Since the imaginal discs originate as distinct anterior and posterior founder populations which flank the parasegment boundary in the embryo, we have suggested that allocation of these cells may depend on intercellular signals transmitted by the secreted Wingless (Wg) protein (Simcox et al., 1989; Cohen, 1990). Wg protein can be detected over a range of 2-3 cells flanking the cells that express *wg* transcript (van den Heuvel et al., 1989; González et al., 1991). *Dll* is expressed in a cluster of cells which is symmetrically distributed around the row of cells expressing *wg* (Cohen,

1990). This is precisely the distribution that one would expect if the secreted Wg protein provided a spatially localized intercellular signal required for activation of *Dll*. Simcox et al. (1989) have shown that specification of the thoracic imaginal primordia also depends on *wg* activity. By using mutations in segment polarity genes to manipulate the anterior-posterior pattern of the embryonic segment, it has been possible to show a compelling correlation between the pattern of *wg* and *Dll* expression in the segment and the resulting anterior-posterior pattern of the leg discs (Simcox et al., 1989; Cohen, 1990). This correlation suggests that early expression of *Dll* responds to the same positional signals that specify the identity of the presumptive imaginal cells. As such, *Dll* expression may provide a useful marker with which to monitor the allocation process.

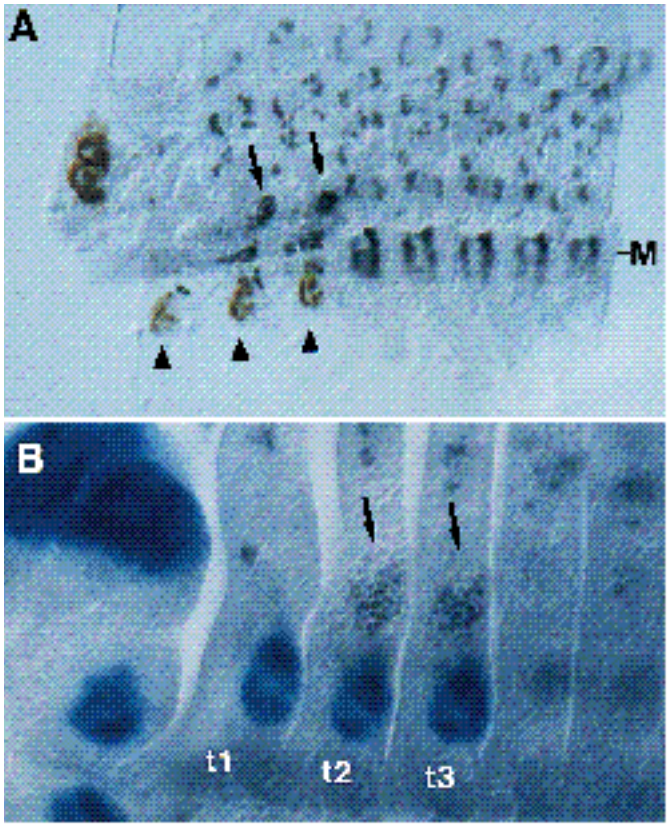

Fig. 1. Molecular markers for the leg and wing primordia.(A) Late embryo (approx. stage 15) carrying the *Dll*-215 late enhancer transgene. The embryo was double labelled with antibodies to vestigial (Vg) protein and anti-β-gal. The pattern of Vg-expressing cells (grey) is complex in the late embryo. The wing and haltere imaginal discs can be seen as sacs of cells invaginating from the ectoderm in the second and third thoracic segments (arrows). Vg also labels several sets of larval muscles and the CNS (not shown). The leg discs (arrowheads) express β-gal protein under control of the *Dll* enhancer (brown). A band of muscle running between the leg discs and the wing and haltere discs is labelled (M). This Fig. is shown to indicate the relative locations of the dorsal and ventral discs primordia late in embryogenesis (for comparison with Fig. 7). (B) Germ band retracted embryo (late stage 12) double labelled for β-gal activity (blue) and by antibody to Vg. The leg primordia in the thoracic segments (t1-t3) are labelled by β-gal expression. The primordia of the wing and haltere discs express Vg (grey, arrows). These clusters are separated from the leg primordia by only about 3-5 cells at their nearest points.

Dll is expressed in clusters of cells surrounding the ends of the ventromedial *wingless* stripes in the trunk segments of 5-5.5-hour old embryos (Cohen, 1990). The observation that *Dll* expression is restricted to a well defined position on the *wg* stripe suggests that a dorsal-ventral positional cue is required in addition to the Wg signal. Based on its pattern of expression, the *decapentaplegic* (*dpp*) gene is a good candidate to provide the second signal. *dpp* encodes a secreted signalling molecule of the TGF-β family (Padgett et al., 1987). *dpp* is expressed in longitudinal stripes running the length of the embryonic trunk region (St. Johnston and Gelbart, 1987; Blackman et al., 1991) at the time when *Dll* is first expressed. Double-labelling studies show that the rows of cells that express *wg* and *dpp* abut one another in each segment, forming a ladder-like pattern (Fig. 2A,B).

In order to trace the early stages of development of the disc primordia we have made use of an early-acting *Dll cis*-regulatory control element (known as *Dll*-304) to direct expression of a *lacZ* reporter gene in the leg primordia. Double-labelling studies show that the *Dll*-304 transgene is expressed as early as the endogenous *Dll* gene and in a spatially correct pattern (see Vachon et al., 1992 and Fig. 6A). The *Dll*-304 transgene is activated in clusters of cells which overlap the intersection points between the two rows of cells expressing *wg* and *dpp* (Fig. 2C). The *Dll*-304 transgene is activated in cells near the intersection point that express *wg* or *dpp* and also in nearby cells that do not express either of these genes. Although the *Dll* enhancer is activated symmetrically about the stripe of *wg*-expressing cells, the cluster is somewhat wedge shaped. At the dorsal side, cells farther from the stripe of *wg*-expressing cells activate the enhancer. These cells are closer to the *dpp*-expressing cells (which are adjacent to the *wg*-expressing cells but which do not overlap them). Similarly, cells closer to the source of the *wg* signal on the ventral side can be farther from the stripe of cells expressing *dpp*. We observe a similar, though less pronounced wedge shape in the pattern of expression of the endogenous Dll protein (Fig. 2D).

The spatial relationship between the cluster of cells in which the *Dll* enhancer is activated and the locations of the cells expressing *wg* and *dpp* suggests that cells that sense adequate concentrations of both secreted proteins might be signalled to activate the early enhancer. Thus the rows of cells that express *wg* and the rows of cells that express *dpp* might be viewed as part of an orthogonal co-ordinate system used to position the leg primordia with respect to anterior-posterior and dorsal-ventral pattern in the segment.

Activation of the early *Dll* enhancer correlates with the temporal requirement for wingless activity in allocation of the imaginal primordia

The early *Dll* enhancer element is only transiently activated in the leg primordia. Although the endogenous *Dll* gene continues to be expressed in the leg primordia throughout embryogenesis (Fig. 3A,B), the *lacZ* RNA encoded by the transgene decays during germ band retraction (Fig. 3C,D).

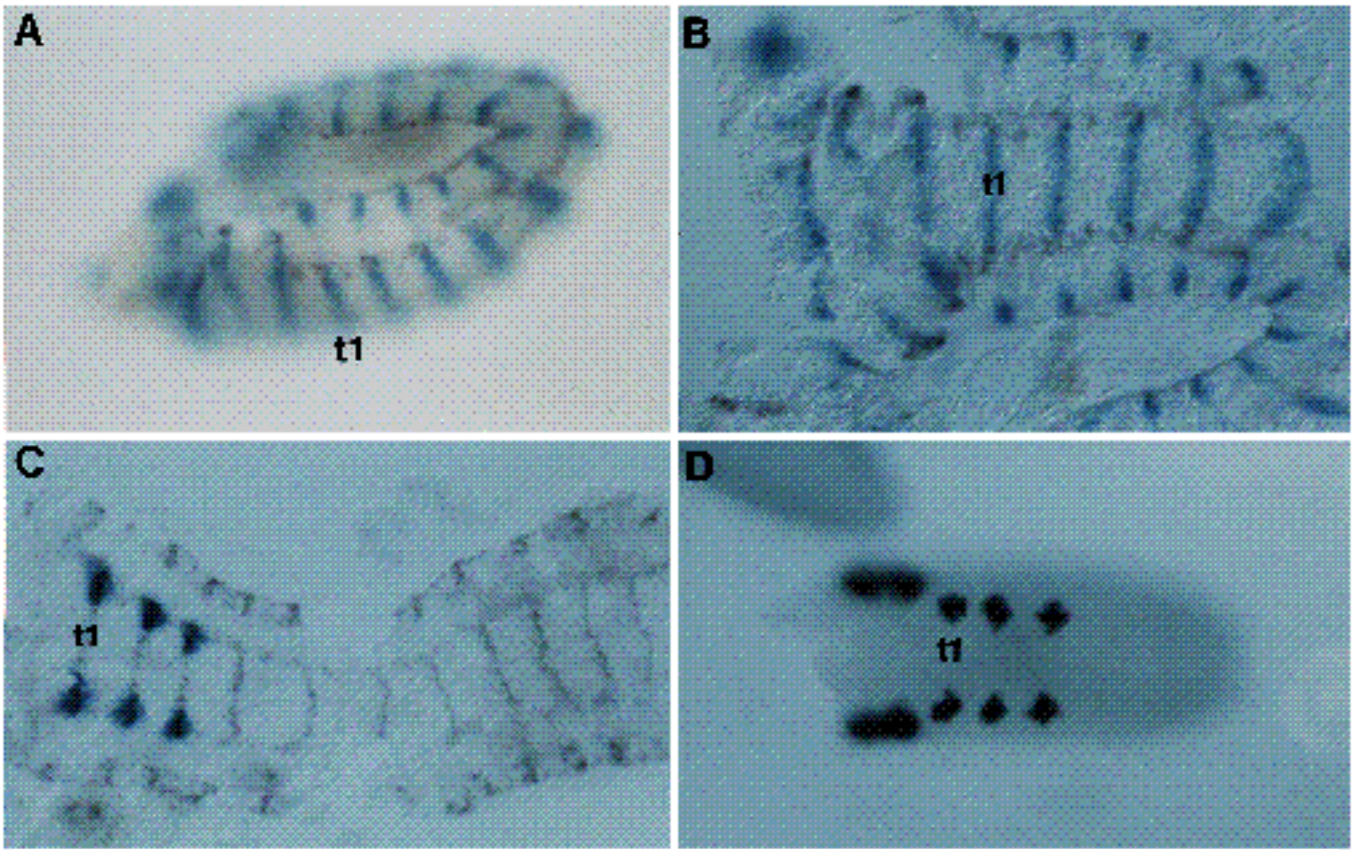

Fig. 2. *Dll* expression labels a population of cells located at the intersection of *wingless* and *dpp* stripes in the embryo. (A) Whole-mount embryo doubly labelled to visualize *dpp* mRNA (brown) and β-gal in cells that express the *wg* gene (blue). *dpp* mRNA is visualized by in situ hybridization and *wg* expression is visualized by histochemical staining for β-gal activity (directed by an enhancer detector insert at the *wg* locus; Perrimon et al., 1991). The longitudinal stripes of cells expressing *dpp* abut the stripes of cells that express *wg* in both the ventrolateral ectoderm and at the dorsal margin of the ectoderm. (B) Dissected embryo displayed to show the ladder-like arrangement of the two sets of stripes. (C) Dissected preparation of an embryo triply labelled to visualize both *wg* and *dpp* mRNAs by in situ hybridization (brown ladder) and the activity of the early *Dll*-304 enhancer element by histochemical staining for β-gal activity. Note that the enhancer is activated in cells that express *wg* and *dpp* as well as in nearby cells that do not. (D) ventral view of a comparably staged embryo stained with antibody to Dll protein. t1 denotes the first thoracic segment.

Like the endogenous gene, expression of the *Dll* early enhancer transgene is *wg*-dependent (data not shown). The observation that the early enhancer is only transiently active raised the possibility that *Dll* expression in the embryo might depend on *wg* only during the time when the early enhancer was functional. To test this proposal we used the temperature-sensitive allele, wg^{IL114} to assess the effects of functionally inactivating Wg protein at different stages during specification of the imaginal primordia. The Wg protein encoded by the wg^{IL114} allele has normal activity at the permissive temperature (18°C) and appears to have no activity at the restrictive temperature (29°C; Baker, 1988; Heemskerk et al., 1991; Bejsovec and Martinez-Arias, 1991). wg^{IL114} embryos were allowed to develop at 18°C and were shifted to the restrictive temperature at intervals before, during and after the activation of the *Dll* early enhancer. *Dll* is not expressed in the leg primordia of wg^{IL114} embryos raised continuously at the restrictive temperature (not shown).

To identify the latest stage at which *Dll* expression in the leg primordia remained *wg*-dependent, we staged the embryos with respect to their pattern of *Dll* expression at the time of the shift to the restrictive temperature. A subset of the embryos in each collection were fixed when their siblings were shifted to the restrictive temperature. Embryos allowed to develop for 7-8 hours at 18°C had not begun to express Dll protein in the leg primordia (Fig. 4A). Mutant embryos from this population failed to express Dll in the legs when tested 3 hours after *wg* activity was removed by the shift to the restrictive temperature (Fig. 4B). Development proceeds normally and *Dll* is activated in the legs of the wg^{+} sibling embryos (Fig. 4C). We conclude that removing *wg* activity at any time prior to the activation of *Dll* blocks subsequent *Dll* expression.

Embryos from a slightly older pool had, on average, just begun to express Dll in the leg primordia (Fig. 4D, arrows; these embryos were allowed to develop for 8-9 hours at 18°C, equivalent to 5-5.5 hours at 25°C). Mutant embryos shifted to the restrictive temperature at this stage are less sensitive to removal of *wg* activity, and continue to show

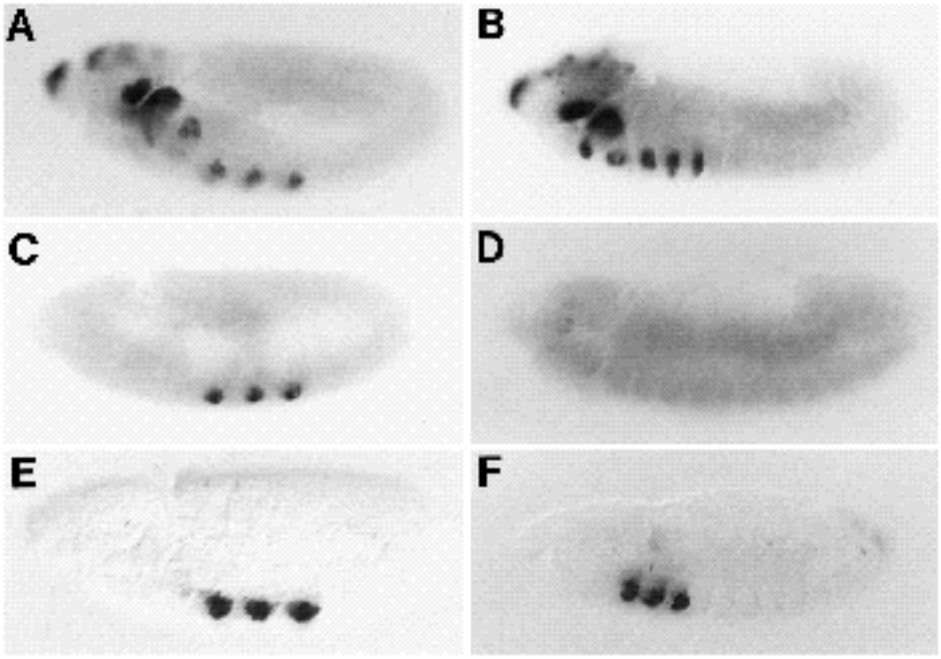

Fig. 3. Comparison of *Dll* RNA, *Dll*-304-*lacZ* RNA and β-gal protein stability. (A,B) *Dll* mRNA and (C,D) *Dll*-304-*lacZ* RNA visualized by whole-mount in situ hybridization in germ band extended and germ band retracted embryos. The transgene RNA decays during germ band retraction indicating that the *Dll*-304 enhancer is only transiently activated. Only a small proportion of embryos continue to express detectable quantities of the transgene RNA by the end of stage 12. The endogenous *Dll* gene continues to be expressed. (E,F) β-gal protein encoded by the transgene RNA, visualized by antibody staining in germ band extended and germ band retracted embryos. β-gal protein is stable and persists long after *lacZ* RNA has decayed.

some Dll expression at later stages. Within this tightly staged population there is a discernible difference between the oldest and youngest embryos. The youngest mutant embryos in the population show no *Dll* expression (as in the younger population described above). We could not detect any difference between the oldest mutant embryos in the population and their wg^+ siblings (these embryos had completed germ band retraction at the time of fixation). Embryos of intermediate age had not quite finished germ band retraction at the time of fixation and showed reduced level of *Dll* expression (arrows, Fig. 4E; comparably aged control sibling, Fig. 4F). The embryo in E is typical of the oldest mutant embryos in which we could detect a significant reduction of Dll expression. Based on the age distribution in the population at the time of the shift to the restrictive temperature, we conclude that these embryos had just begun to detectably express Dll protein in the leg primordia when Wg protein was inactivated. Once Dll protein is present, there is little effect of removing *wg* function.

Dll remains dependent on *wg* activity until the early enhancer has been activated and then rapidly becomes independent of further signalling from *wg.* Interestingly, Baker (1988) has shown that *wg* activity is not required until 4-5 hours of embryogenesis to specify the Keilin's organs (and therefore presumably *Dll* expression, since development of the Keilin's organs depends on *Dll* gene activity; Cohen and Jürgens, 1989). These observations suggest that the signal to activate *Dll* is transmitted during a relatively brief time interval at about 5-5.5 hours of embryogenesis. The time interval, during which the Wg signal appears to be transmitted to *Dll,* correlates well with the time at which the *wg* and *dpp* expressing cells are arranged in the ladder-like pattern.

Does the temporal requirement for *wg* activity in activating *Dll* correspond to the temporal requirement for *wg* in allocation of the imaginal primordia? We made use of a molecular probe from the *escargot* (*esg*) gene to monitor the development of the imaginal primordia in a second set of wingless temperature shift experiments. *esg* transcript is expressed in imaginal cells of the thoracic, genital and cephalic discs beginning at stage 13 of embryogenesis (Whiteley et al., 1992; Hartenstein and Jan, 1992). The specification of the thoracic imaginal cells shows precisely the same temporal dependence on *wg* activity that we observed for Dll expression. Embryos shifted to the restrictive temperature before 5 hours (25°C) lack thoracic imaginal disc primordia (Fig. 4G). Their Wg^+ siblings show well developed dorsal and ventral thoracic discs (Fig. 4H). The genital and cephalic disc primordia appear to be unaffected by removal of *wg* activity. The embryos in this population were of the same age as the embryos in Fig. 4A-C at the time of the temperature shift. Embryos shifted to the restrictive temperature after Dll was activated in the legs were insensitive to the removal of *wg* activity and developed normal thoracic discs (assessed by *esg* staining, data not shown).

These observations suggest that the dorsal and ventral thoracic imaginal primordia are allocated in response to a *wg*-dependent signal at approximately 5 hours of embryogenesis. They also suggest that expression of Dll in the leg primordia may be initiated in response to the same signals that are responsible for allocation of the imaginal primordia. Additional support for this suggestion derives from following the developmental fate of cells that first express *Dll* in response to the *wg* signal.

A group of cells leaves the leg primordium during germ band retraction

The ladder-like arrangement of cells expressing *wg* and *dpp* only exists for a relatively short time in the germ band extended embryo. During germ band retraction the row of

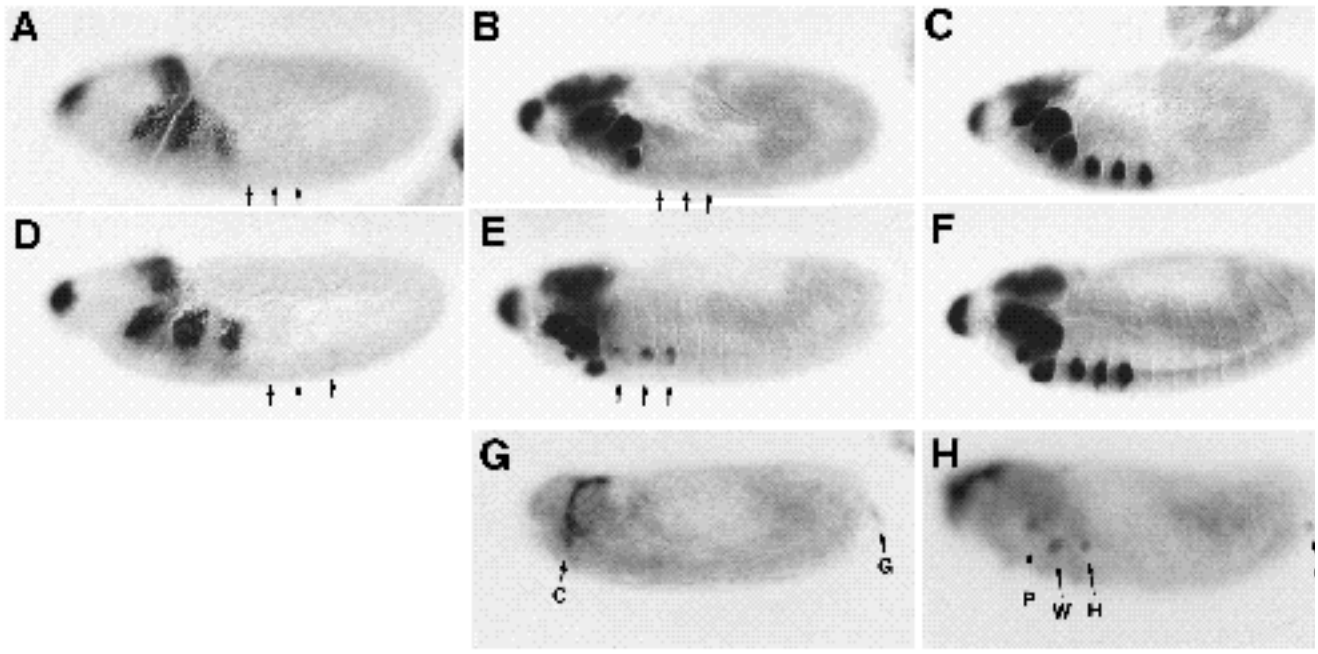

Fig. 4. *Dll* expression and allocation of the imaginal primordia depend on *wingless* activity at about 5 hours of embryogenesis. (A-C) Embryos allowed to develop at 18°C for 7-8 hours at 18°C before the shift to the restrictive temperature. (A) Representative embryo fixed at the time of the shift to 29°C. Dll protein is not yet detectable in the leg primordia of these embryos (arrows). (B) wg^{IL114} mutant embryo fixed 3 hours after shift to 29°C. Dll is absent from the leg primordia (arrows), although it continues to be expressed in the head. (C) Dll is abundant in the leg primordia of a heterozygous sibling embryo (of comparable age to the mutant in C). (D-F) Embryos allowed to develop at 18°C for 8-9 hours at 18°C before the shift to the restrictive temperature. (D) Dll protein is just becoming detectable in the leg primordia of embryos fixed at the time of the shift to 29°C (arrows). (The appearance of the endogenous Dll protein is somewhat delayed with respect to the transcriptional activation of the *Dll*-304 element. β-gal protein expressed by *Dll*-304 is detectable earlier than the endogenous Dll protein in double-labelling experiments, data not shown). (E) wg^{IL114} mutant from this pool fixed 3 hours after shift to 29°C. This embryo is typical of the oldest affected mutant embryos that we observed. The level of Dll protein is much reduced in the legs of the mutant, as compared to their heterozygous siblings (shown in F). No difference could be detected between mutant and wild-type embryos that were older than the embryo shown in E (i.e. fully germ band retracted). Since the oldest mutant embryos in the population no longer show any defect in *Dll* expression, the proportion of affected embryos is smaller in the group shifted between 8-9 hours than in the group shifted between 7-8 hours. *wg* activity appears to be required until *Dll* is turned on via the early enhancer. (G,H) Embryos were collected, aged and shifted to the restrictive temperature as in A-C. The age at the time of the shift to 29°C was verified by Dll expression (not shown). The embryos were aged for 7 hours at 29°C and labelled to visualize *escargot* RNA by in situ hybridization. (G) wg^{IL114} mutant embryos from this pool lack dorsal and ventral thoracic discs. The genital (G) and cephalic (C) imaginal primordia are not affected. (H) Heterozygous sibling embryo. The plane of focus in on the dorsal thorax to show the prothoracic (P), wing (W) and haltere (H) discs. The prothoracic disc is associated with the anterior spiracle. The leg discs are visible, but out of focus, at the ventral edge of the embryo in the thoracic segments. Note that head involution fails in the mutant embryos, so the cephalic imaginal cells have not migrated as far to the anterior in G, as in the control sibling in H.

cells that express *dpp* is displaced dorsally, away from the *wg* stripe (Fig. 5A,B). As this occurs, a subset of the cells, which expressed β-gal protein under control of the early *Dll* enhancer (β-gal$^+$ cells), also separate from the ventral cluster. The dorsal-most group of these cells appear to remain aligned with the cells that express *dpp* in the longitudinal stripe (black arrow, Fig. 5B), while the ventral cluster remain centered on the end of the wingless stripe (white arrow, Fig. 5B). For comparison, a double label showing the location of the leg primordia and the dorsally displaced stripe of *dpp* expression is shown in Fig. 5C. The leg primordia are labelled by β-gal expression directed by the late acting *Dll* enhancer transgene (blue). The late acting *Dll* enhancer is turned on somewhat after the early enhancer in cells of the leg primordia (which initially express *Dll* under control of the early enhancer). Note that cells labelled later do not show the extensive dorsal displacement seen in the cells that are labelled at an earlier stage by expression of the early enhancer.

The dorsal displacement of β-gal$^+$ cells is not a reflection of the normal pattern of *lacZ* mRNA expression directed by the early enhancer. β-gal protein is quite stable and persists in these cells long after the *lacZ* mRNA has decayed (see Fig. 3C-F for a comparison of the stability of *lacZ* RNA and β-gal protein). The β-gal$^+$ cells in the dorsal extension must have originated in the ventral cluster of cells that expressed the *Dll*-304 transgene in the germ band extended embryo. The perdurance of β-gal protein provides a long-lived molecular label which allows us to observe the dorsal displacement of these cells.

To assess the relationship between the β-gal$^+$ cells (in which the early enhancer was active) and the cells of the

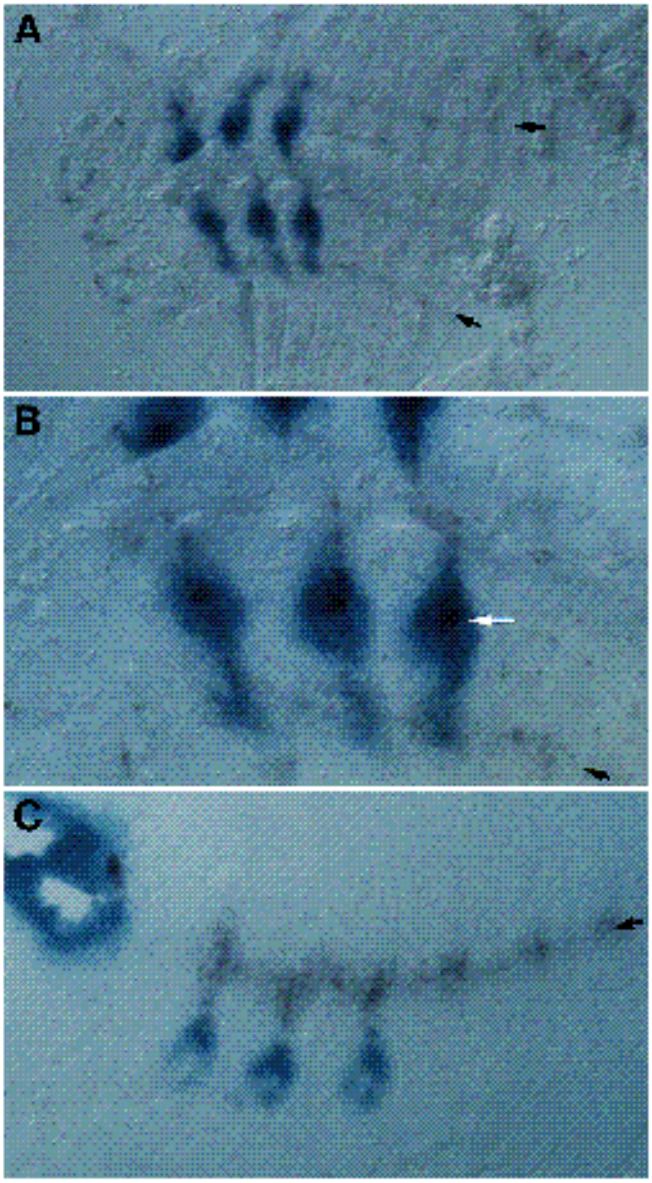

Fig. 5. *Dll*-β-gal expressing cells leave the leg primordium during germ band retraction. (A) Germ band retracted embryo triply labelled as in Fig. 2C to visualize cells which expressed the early *Dll* enhancer at germ band extension, as well as cells expressing *dpp* and *wg*. As the embryo retracts its germ band the dpp-expressing cells move dorsally and separate from the wg-expressing cells, so that the ladder-like arrangement shown in 2C is disrupted. A subset of the cells, which expressed the early *Dll* enhancer, move dorsally in register with the *dpp* stripe (black arrow). The embryo has been dissected open so that the ventral midline is in the center of the picture and dorsal is both up and down. Anterior is to the left. (B) Detail of the lower part of A showing the end of the ventromedial *wg* stripe as a darker spot within the larger ventral cluster of β-gal$^+$ cells (white arrow). Note that the β-gal protein visualized in A and B reflects the prior expression of the transgene in these cells when they were located in the ventral cluster, as shown in Fig. 2C. No *lacZ* RNA is detectable in the dorsal extension of β-gal$^+$ cells at this stage (Fig. 3). Dorsal is down and anterior is to the left. (C) Comparably staged embryo labelled to visualize *dpp* mRNA (brown) and the activity of the late *Dll*-215 enhancer in the leg and cephalic limb primordia (blue). In C dorsal is up and anterior is to the left.

leg primordium, we compared the expression of β-gal in these cells with that of the endogenous Dll protein by double-label immunofluorescence (Fig. 6). As expected from the similarity in the patterns of *lacZ* and *Dll* mRNAs at early stages, the initial patterns of *Dll*-304 β-gal and Dll proteins are essentially superimposable in the leg primordia of the germ band extended embryo (Vachon et al., 1992, and Fig. 6A). As described above, cells marked by *Dll*-304 β-gal protein (green, Fig. 6B) are displaced dorsally, while cells expressing the endogenous Dll protein (red) remain in a tightly localized ventral cluster. The overlap between the endogenous Dll protein and β-gal gives the cells of the ventral cluster a yellow appearance, while the dorsally moving β-gal$^+$ cells, which have left the leg cluster, appear green.

As the germ band shortens the segments become narrower and elongate dorsally. This process continues until dorsal closure is completed. The dorsal displacement of β-gal$^+$ cells may reflect in part the change in shape of the segment during germ band retraction. Note however, that the shape of the cluster of β-gal$^+$ cells that move dorsally is not easily explained by a bulk shift of cells in the segment (i.e. it does not appear to be a simple dorsal broadening of the ventral cluster of β-gal$^+$ cells). Rather, the displacement of labelled cells is observed predominantly in the posterior half of the segment. By following incorporation of bromo-deoxyuridine into dividing cells, Bate and Martinez-Arias (1991) have reported that the region between the leg and wing discs shows a locally high level of proliferation between 5 and 11 hours of embryogenesis. The posterior region of the segment shows strongest labelling, consistent with a role for local cell division in the dorsal displacement of β-gal$^+$ cells in the posterior part of the segment.

Wing and haltere precursor cells derive from the ventral cluster

The dorsal-most group of the displaced β-gal$^+$ cells come to lie the region in which the wing and haltere imaginal disc primordia can first be morphologically detected. As shown in Fig. 1, the invaginating discs have a distinctive appearance and can be readily identified in flattened preparations of approx. 12-hour old embryos. On morphological grounds, the dorsal-most group of β-gal$^+$ cells appear to invaginate to form the wing and haltere imaginal discs (Fig. 6C). To confirm that the β-gal$^+$ cells were in fact contributing to the forming dorsal discs we performed double labelling using antibodies to Vg protein and β-gal (Fig. 7). Vg (red) and β-gal (green) proteins are co-expressed in the invaginating wing and haltere discs of transgenic embryos, which therefore appear yellow. These observations indicate that cells that contribute to the formation of the dorsal imaginal discs expressed the *Dll* early enhancer transgene some hours earlier in the germ band extended embryo. These cells appear to originate together with the cells of the presumptive leg in a ventrally located cluster of cells in which the *Dll* early enhancer element is active. A subset of the presumptive imaginal cells are displaced dorsally. After separating from the ventral cluster, these cells begin to express Vg and subsequently invaginate to form the wing and haltere discs.

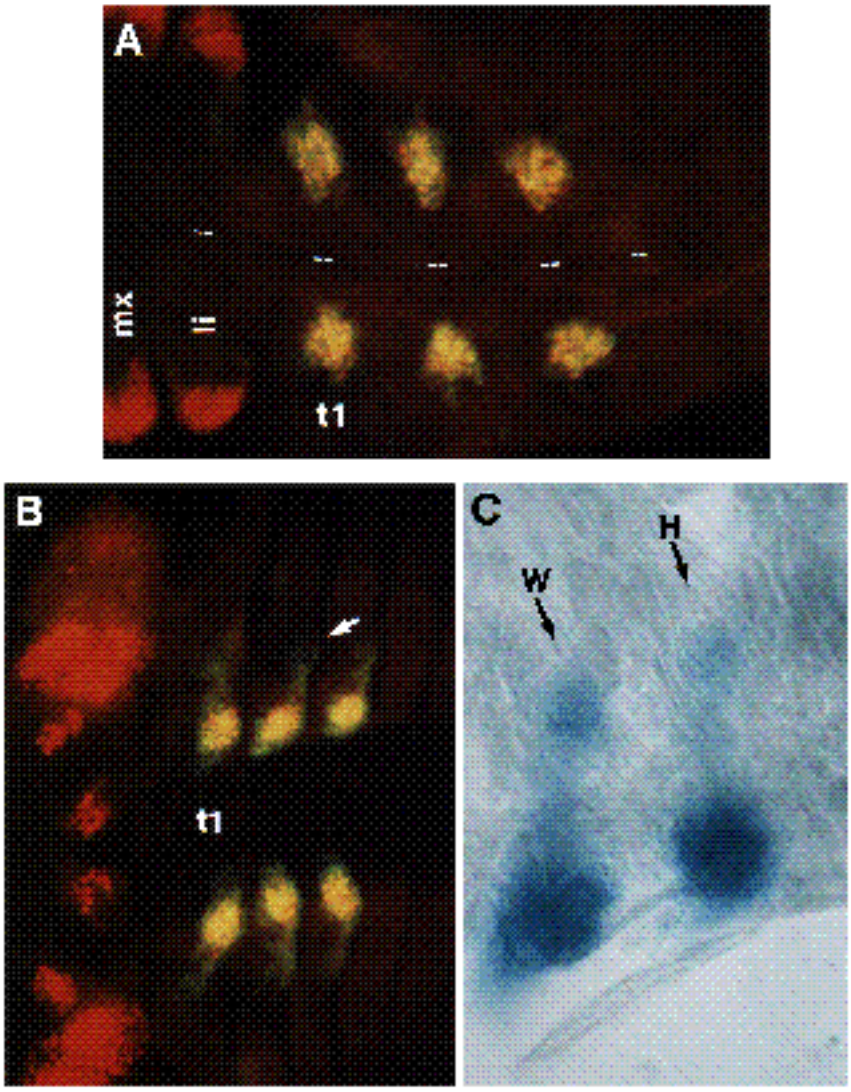

Fig. 6. *Dll*-β-gal expressing cells move to the location of the dorsal discs. (A, B) Embryos were labelled to visualize Dll protein (red) and β-gal protein (green) by double immunofluorescence. Cells expressing both proteins appear yellow. Embryos have been dissected open and flattened so that the ventral midline lies in the center of the photograph (indicated by dashes in A). Anterior is to the left. (A) The initial pattern of β-gal protein expression is virtually superimposable on the pattern of expression of the endogenous Dll protein in the thoracic segments of a stage 11 embryo. Dll protein is nuclear. β-gal protein is cytoplasmic. All of the Dll-expressing cells in t1-t3 express β-gal protein. At most 1 or 2 cells express β-gal but not Dll in each leg primordium. The transgene is not detectably expressed in the maxillary (mx) or labial (li) segments. The early overlap of Dll and β-gal expressing cells has been described in Vachon et al. (1992) and is shown here only to define the starting point for comparison with the later stage shown in B. (B) During stage 12, some β-gal expressing cells move out of the leg primordium. These cells lose expression of Dll and so appear green (e.g. arrow in t2). (C) Detail of an embryo (approx. stage 15) doubly labelled for β-gal activity by histochemical staining (blue) and by antibody to Dll protein (black). A subset of β-gal$^+$ cells has migrated dorsally and begun to invaginate in the location of the wing and haltere discs (W, H). These cells are smaller and rounded, and appear blue against the grey background stain. Stalks of β-gal$^+$ cells can be seen (out of the plane of focus) to connect the second leg disc with the wing and the 3rd leg disc with the haltere.

Dll is not required for specification of the imaginal primordia

The observation that both dorsal and ventral disc primordia develop from cells that expressed the *Dll* early enhancer raised the possibility that *Dll* plays a role in allocation of the imaginal primordia. To address this issue, *Dll* null mutant embryos were cultured in the abdomens of female flies by transplantation of embryo fragments. In vivo culture allows the imaginal discs to be rescued from embryos carrying lethal mutations, if the mutation does not interfere with growth of the disc cells (as described in Simcox et al., 1989). *Dll* null mutant embryos produced both leg and wing imaginal discs following in vivo culture, indicating that although the discs originate from a population of *Dll*-expressing cells in the embryo, *Dll* gene activity is not required for the allocation of the disc primordia.

To assess their developmental potential, *Dll* mutant imaginal discs were transplanted into wild-type larvae and induced to undergo metamorphosis. Leg and wing discs were recovered with equal frequency from wild-type and from *Dll* null-mutant embryos following embryo culture (Table 1). Leg discs from *Dll* mutant embryos produced structures characteristic of the ventral thoracic body wall and coxa. No structures from the trochanter, femur, tibia or tarsal segments of the leg were recovered from mutant discs (Fig. 8). Control discs, cultured from wild-type embryos, produced a range of structures characteristic of all of the major leg segments (Table 1). These findings are consistent with previous clonal analysis (Cohen and Jürgens, 1989) and indicate that *Dll* is required in only a subset of cells in the leg disc primordium to distinguish the presumptive leg cells from the surrounding body wall.

DISCUSSION

When does allocation of the imaginal progenitor cells occur?

Early studies on the determination of the imaginal cells in the embryo suggested that the imaginal primordia are determined at the blastoderm stage (Chan and Gehring, 1971;

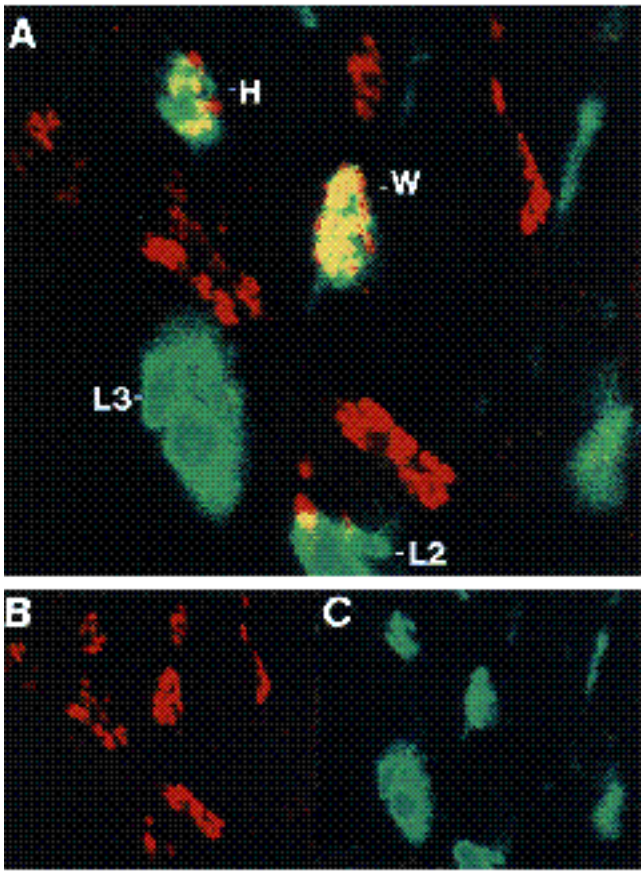

Fig. 7. *Dll*-β-gal expressing cells form the wing and haltere discs**.** (A) Immunofluorescent localization of β-gal (green) and vestigial (red) expressing cells in a late embryo imaged using a confocal microscope. The invaginated wing (W) and haltere (H) discs contain both proteins and appear yellow. The second (L2) and third leg (L3) discs contain β-gal and label green. Note the band of muscle cells (red) between the legs and wing/haltere discs (compare with Fig. 1). B and C show the individual images of the Vg and β-gal labellings that were merged to make the image in A.

Table 1. Structures produced by *Dll* mutant leg imaginal discs

	Number (%)			
Genotype (n)	Wing	Ventral body wall including coxa	Trochanter	Distal leg
Dll⁻/*Dll*⁻ (11)	9 (82)	10 (90)	0 (0)	0 (0)
+/+ (5)	4 (80)	4 (80)	4 (80)	5 (100)

The different regions of the leg can be characterized by the types of sensory structures that they produce. These have been described in detail for the first leg (11). Although different in some details, the overall pattern of sensory structures for second and third leg are similar. Groups of sensilla trichodea (st) are found on the coxa (st8, st4, st3) and on the trochanter (GST, st5, st1). The trochanter also has groups of sensilla campaniforma (sc) which are not found on the coxa. Distal leg segments are most easily characterized by the presence of bracted bristles, and in some cases by the claw. Ventral body wall and coxa were identified by the presence of sternopleural bristles (for T2 legs), st8, st4 and st3 (for T1) groups and the absenceof sc groups. Trochanter was identified by the presence of 2 GST groups, sc3, a large sc group, st1 and st5. In the *Dll* mutant legs coxa structures were clearly identified in all cases. No GST groups, sensilla campaniforma groups, or bracted bristles were seen indicating the absence trochanter and distal leg structures. Bracted bristles were seen in all control legs indicating the presence of distal leg segments. In 4 of the control legs characteristic coxa and trochanter structures were identified. Although st groups were identifiable in the 5th control leg, it was not possible to score the more proximal leg structures unambiguously because the proximal segments of the disc were obscured by more distal tissue.

Simcox and Sang, 1983). However, a recent set of experiments by Meise and Janning provides compelling evidence to the contrary. Meise and Janning (1991) transplanted single blastoderm cells from a donor embryo, which expressed β-gal in all cells, into a wild-type host embryo at the blastoderm stage and monitored the fate of the clonal progeny of the injected cell. A single cell injected at the blastoderm stage can contribute to both an imaginal disc and to the larval hypoderm. These experiments clearly demonstrate that there is no lineage restriction between larval and imaginal ectoderm at the blastoderm stage, and therefore indicate that the imaginal cells cannot have been allocated at the blastoderm stage.

The formation of imaginal primordia in the thoracic segments requires the function of the *wingless* gene (Simcox et al., 1989). Using a temperature-sensitive *wg* mutation, we have shown that *wg* activity is required at approximately 5-5.5 hours of embryogenesis for the subsequent development of the discs. At this time *wg* and *dpp* are expressed in a ladder-like arrangement in the ectoderm. We suggest that allocation of the thoracic imaginal progenitor populations occurs in groups of cells in response to the combined intercellular signals conveyed by the secreted Wg and Dpp proteins (Fig. 9). Although *Dll* expression appears to mark the location of the nascent thoracic imaginal disc primordia, we have shown that *Dll* gene function is not required for allocation of the imaginal cells. *Dll* is required for the development of the larval leg in the embryo, and as shown here, in only a subset of leg imaginal disc cells. Thus specification of the identity of the disc primordium may represent an independent response to the positional signals that turn on *Dll* in the larval leg. This implies that some gene(s) other than *Dll* must be activated in response to *wingless* and a dorsal-ventral positional cue (which may be encoded by *dpp*) in the germ band extended embryo.

Does *dpp* co-operate with wg in specification of the thoracic imaginal primordia?

On theoretical grounds, Meinhardt (1983) proposed that the imaginal disc primordia would be established in a naive field of cells in response to a secreted morphogen. He proposed that localized production of the morphogen would result from interaction between cells that derive from adjacent compartments in the embryonic segment. A more recent version of this model invokes the overlap between a secreted A/P morphogen and a secreted D/V morphogen to specify the limb field (Meinhardt, 1991). Our data suggest that the relevant positional information is transmitted by the combined input from two molecularly distinct intercellular signals, the Wingless and Dpp proteins. These observations are compatible with the basic premise of Meinhardt's model that the interaction between cells with different properties would provide the signal to allocate the imaginal primordia.

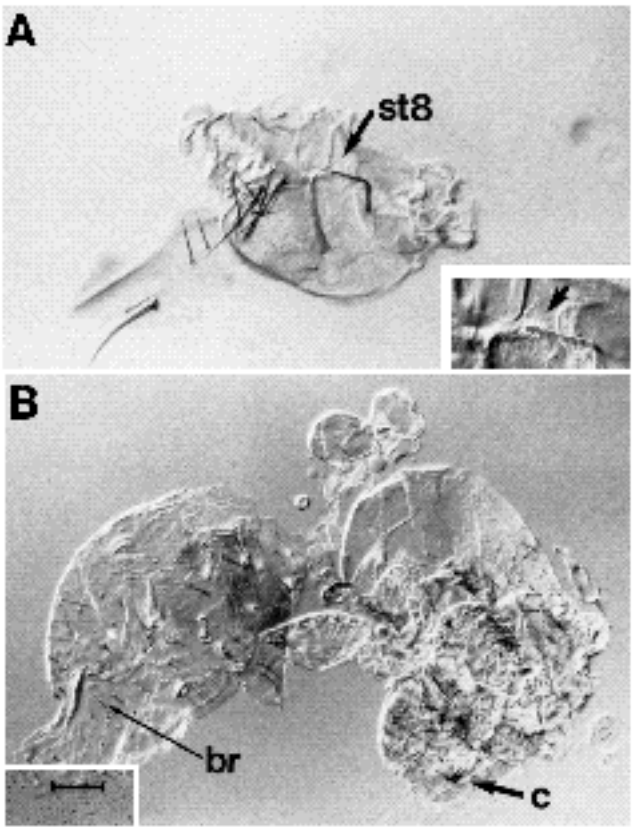

Fig. 8. Leg discs derived from *Dll* mutant and wild-type embryos after in vivo culture and transplantation. (A) A paired first thoracic leg disc recovered after in vivo culture and transplantation of a complete lack-of-function *Dll* mutant embryo. The mutant discs are small and only produce structures characteristic of the ventral body wall and coxa. The location of a sensilla trichodea st8 group is indicated by the arrow. The inset shows this st8 group at higher magnification. This particular disc also contains st4, st3 and BH^- groups characteristic of the coxa of the first thoracic leg (Schubiger, 1968). No structures characteristic of the trochanter or distal leg segments were observed. (B) Cultured leg disc from a wild-type control. The cultured discs are recovered as inverted vesicles, so the normal organization of the disc is obscured. Control discs always produce bracted bristles (br), which are characteristic of the distal leg segments. In this case, the most extreme distal structure, the claws (c) can be seen. Scale bar = 80 μm in A and B.

We have shown previously that the (secreted) *wg* signal is required for *Dll* expression and specification of the imaginal primordia (Simcox et al., 1989; Cohen, 1990). Similarly, *dpp* activity is required for expression of *Dll* in the leg primordia (B.C. unpublished data). However, since *dpp* null mutants cause dramatic ventralization of the embryo, we cannot yet determine whether the absence of *Dll* expression reflects a direct requirement for *dpp* in activating *Dll* or whether the disc primordia have indirectly been shifted off the fate map (Irish and Gelbart, 1987; Ray et al., 1991). Like *dpp, tolloid* is required for dorsal-ventral patterning and encodes a member of the TGF-β family (Shimell et al., 1991). *tolloid* expression overlaps *dpp* in the lateral stripes which make up the ladder. However, *tolloid* function is not required for activation of *Dll* in the imaginal primordia (data not shown).

Although we do not yet know whether *dpp* serves as the direct source of a positional signal, the combination of the Wnt and TGF-β (or other) signals is, in principle, sufficient to specify the identity of the leg precursor cells. The use of two secreted signalling systems would provide an elegant mechanism with which to allocate groups of cells to the disc founder populations, based on their locations in the embryonic ectoderm. There is no reason *a priori* why the second 'line' need be encoded by a secreted signalling molecule. However, we favor this proposal because of the detailed spatial relationship between cells expressing *Dll*, *wg* and *dpp*. The use of two secreted signals provides a plausible model to explain the wedge shape of the cluster of cells that activate the early enhancer (Fig. 9). We can account for the wedge shape of the cluster if we assume that a higher concentration of one signal can make up for a slightly reduced level of the other signal. In this way cells close to the source of the *wg* signal require slightly less of the *dpp* signal to activate the enhancer, leading to a narrowing of the cluster toward its ventral side. Similarly cells closer to the source of the *dpp* signal require slightly less of the *wg* signal, leading to a broadening of the cluster along its dorsal side (Fig. 9).

One set of signals for allocation of dorsal and ventral discs

Our description of the origin of the dorsal disc primordia confirms previous genetic studies which suggest that the dorsal and ventral discs originate in close proximity. Using a molecular lineage tag, we have shown that the leg and wing discs originate within a cluster of cells that expressed *Dll* in the early embryo. The cluster of *Dll*-expressing cells is aligned with the intersection of the stripes of *wg* and *dpp*-expressing cells. Formation of both dorsal and ventral discs depends on *wg* activity at this time. As the germ band retracts, the stripes of cells expressing *wg* and *dpp* separate. The presumptive dorsal disc cells remain aligned with the *dpp* stripe during this movement, while the progenitors of the leg disc remain aligned with the end of the ventromedial *wg* stripe (Fig. 9). An appealing feature of this model is that only one set of positional cues is required for allocation of both dorsal and ventral thoracic discs.

Similar observations on the separation of dorsal and ventral discs have been reported by Anderson (1963) from studies on the Queensland fruit fly, *Dacus tyroni*. According to Anderson the wing and haltere discs originate as part of the leg discs, from which they become separated during embryonic development by local cell division. The leg and wing discs are physically connected by a stalk in the mature *Dacus* embryo. Dorsal migration of the wing precursors seems to be typical of all winged insects, not only of dipterans. The wings migrate away from the legs in a dorsal and posterior direction during successive moults in a variety of hemimetabolous insects. In primitive paleo-insects the wing was moved by muscles connected to the leg (see Kukalova-Peck, 1978 for descriptions and discussion).

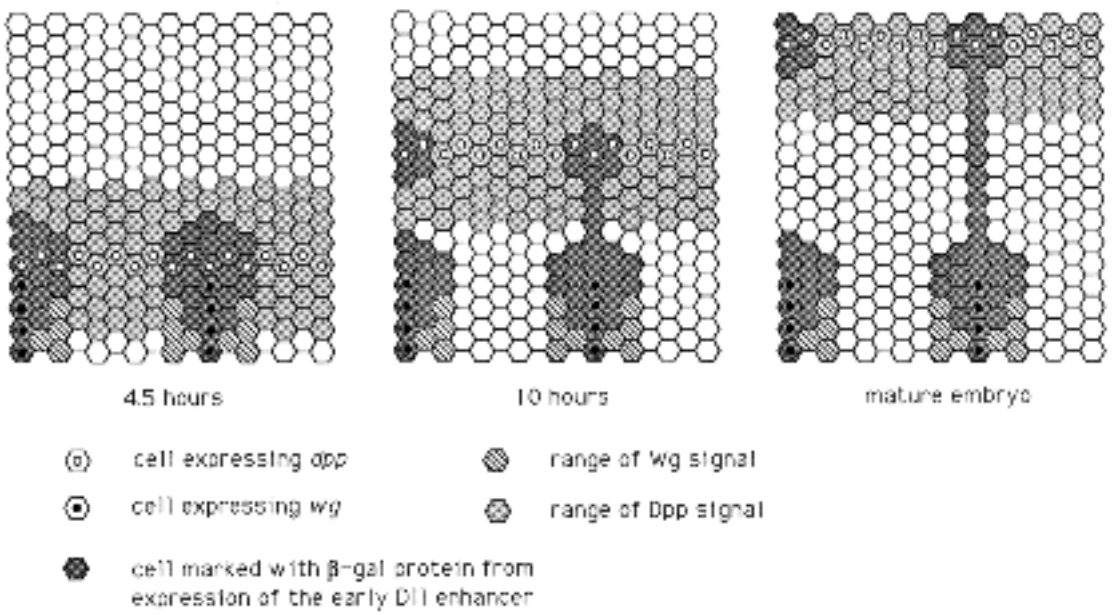

Fig. 9. A model for the allocation and separation of the thoracic imaginal primordia. 5 hours: Schematic representation of the allocation of the imaginal primordia. Lines of cells expressing *wingless* and *dpp* form a ladder-like pattern in the embryo. Wg protein is secreted and can be detected over a range of about 2-3 cells (van den Heuvel et al., 1989, González et al., 1991). Dpp protein is thought to be secreted, for the purposes of the model we assume that the functional range of the secreted Dpp protein is approximately 3 cells. We suggest that groups of cells which lie near the intersections between the sources of the Wg and Dpp signals are signalled to become imaginal progenitor cells. Expression of the early *Dll* enhancer appears to be an independent response to these signals. 10 hours: β-gal protein provides a stable molecular marker that allows us to trace the cells in which the *Dll* enhancer was activated. As the germ band retracts the lines of cells expressing *wg* and *dpp* separate. Cells that expressed the *Dll* enhancer are displaced dorsally in register with the line of cells expressing *dpp*. The dorsal-most cells in this cluster ultimately contribute to the formation of the dorsal discs in the later embryo.

Do leg and wing derive from a common imaginal primordium?

Gynandromorph analysis has suggested that the dorsal and ventral disc primordia originate in very close proximity (Wieschaus and Gehring, 1976a). The observation that the spacing between markers on the two discs can be closer that the separation between some markers within one disc suggests that the primordia derive from an overlapping population of progenitor cells. Are the dorsal and ventral discs genetically distinct entities at the time of their allocation? The data presently available from analysis of genetic mosaics do not allow us to answer this question. The dorsal and ventral discs have been shown to share common progenitors prior to allocation. Clones of cells, marked by X-ray induced mitotic recombination during the cell division following the blastoderm stage, can contribute to both leg and wing disc primordia (Wieschaus and Gehring, 1976b; Steiner, 1976; Lawrence and Morata, 1977). Clones produced one cell division later are restricted to either the dorsal or ventral discs. The limited number of post-blastoderm cell divisions restricts the production of mosaics after allocation of the imaginal cells. Consequently, clonal analysis has not distinguished whether the dorsal and ventral discs originate as a common primordium or as genetically distinct but adjacent primordia.

We have shown that the cluster of cells expressing the early *Dll* enhancer contains imaginal disc progenitor cells for both the dorsal and ventral discs. This observation demonstrates that at least one aspect of the molecular identity of this population appears to be specified by the combination of *wg* and *dpp* signals. We have also shown that it is not possible to distinguish between the time at which *wg* gene activity is required for allocation of the imaginal primordia and for initiation of *Dll* expression. This conclusion does not imply that *Dll* has any role in allocation. Nonetheless, expression of the *Dll* enhancer shows that this population can be signalled as a group to adopt a particular molecular identity. Although we cannot exclude the possibility that there might be genetically distinct dorsal and ventral imaginal sub-primordia within this cluster, we suggest that the simplest explanation for our data and for the genetic mosaic studies lies in a common imaginal primordium.

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