Wnt and TGFβ signals subdivide the AbdA Hox domain during Drosophila mesoderm patterning

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SUMMARY

Hox genes have large expression domains yet control the formation of fine pattern elements at specific locations. We have examined the mechanism underlying subdivision of the abdominal-A (abdA) Hox domain in the visceral mesoderm. AbdA directs formation of an embryonic midgut constriction at a precise location within the broad and uniform abdA expression domain. The constriction divides the abdA domain of the midgut into two chambers, the anterior one producing the Pointed (Pnt) ETS transcription factors and the posterior one the Odd-paired (Opa) zinc finger protein. Transcription of both pnt and opa is activated by abdA but the adjacent non-overlapping patterns are not due to mutual opa-pnt regulation. Near the anterior limit of the abdA domain, two signals, Dpp (a TGFβ) and Wg (a Wnt), are produced, in adjacent non-overlapping patterns, under Hox control in mesoderm cells. The two signals are known to regulate local mesodermal cell fates and to signal to the endoderm. We find that, in addition, they precisely subdivide the abdA domain: Wg acts upon anterior abdA domain cells to activate pnt transcription, while Dpp is essential in the same region to prevent abdA from activating opa transcription. pnt activation is required to determine the appropriate numbers of mesodermal cells in the third midgut chamber.

Key words: Hox, homeotic genes, abdominal-A, pointed, odd paired, wingless, decapentaplegic, Ultrabithorax, Visceral mesoderm, Ets, Drosophila

INTRODUCTION

Hox genes control anterior-posterior cell fates in multiple tissues of organisms as different as flies, mice and worms (reviewed in McGinnis and Krumlauf, 1992; Lawrence and Morata, 1994). The Hox proteins are DNA-binding transcription factors thought to act as master ‘selector’ genes, which regulate arrays of downstream target genes (reviewed in Graba et al., 1997). These target or ‘realizator’ genes carry out specific morphogenetic events to produce characteristic structures along the body axis. The search for Hox downstream targets is currently an important avenue in the investigation of Hox gene function. Few Hox target genes are known in flies and almost none in vertebrates, so the mechanisms by which Hox genes control morphogenesis remain incompletely understood.

Most Hox targets identified to date have been found in the visceral mesoderm (VM) of Drosophila, where Hox genes are required for morphogenesis of three constrictions that divide the embryonic midgut into four approximately equal-sized chambers (reviewed in Bier, 1994; Bilder and Scott 1995). Formation and placement of the midgut constrictions is therefore an indicator of Hox target gene action along the anterior-posterior axis. The well-characterized development of the midgut and the simple expression of Hox genes within the VM have allowed significant progress in understanding how Hox genes regulate their targets to dictate morphogenetic and cell fate decisions. In particular, a hierarchy of genes regulated by the Hox genes Ultrabithorax (Ubx) and abdominal-A (abdA) to form the central midgut constriction has been identified. These molecules include the secreted signalling proteins Wingless (Wg) and Decapentaplegic (Dpp), components involved in transducing Wg and Dpp signals, and the transcription factor Teashirt (Tsh) (reviewed in Skaer, 1993; Yu et al., 1996; Nellen et al., 1994; Penton et al., 1994; Mathies et al., 1994). Wg and Dpp are also required to induce differentiation of a specific cell type, the copper cell, in the underlying endoderm (Hoppler and Bienz, 1994).

The genetic control of the posterior midgut morphogenesis is less well understood, but presents an important problem in Hox-regulated patterning. While the central constriction forms at the interface of the Ubx and abdA domains, the posterior constriction forms at a precise location in the midst of a broad, apparently uniform domain of abdA expression. The posterior constriction divides the posterior midgut into the third and fourth midgut chambers. How do cells within this single domain of Hox gene expression take on third or fourth chamber identities and how is constriction location controlled?

Formation of the posterior constriction requires abdA and the Hox cofactor extradenticle (exd) (Rauskolb and Wiechaus,
1994), but no Hox targets involved in morphogenesis of the posterior midgut have been identified. The best candidate for a target involved in posterior midgut morphogenesis to date is odd-paired (opa), which encodes a zinc-finger protein that requires abdA for its expression in the VM of the fourth midgut chamber (Cimbora and Sakonju, 1995). However, opa mutants are defective in the formation of the VM (Cimbora and Sakonju, 1995; Azpiazu et al., 1996), so it has not been possible to investigate a subsequent role for opa in constriction formation.

Here we describe the identification of pointed (pnt) (Klümpt, 1993) as a target of abdA that is required for morphogenesis of the posterior midgut. pnt is expressed throughout the VM of the third midgut chamber and is required for determining the appropriate number of VM cells in this chamber. The activation of pnt and opa by abdA is modulated by the Hox-regulated diffusible signals Dpp and Wg, which partition the broad domain of abdA expression into pnt- and opa-expressing subdomains that define the third and fourth midgut chambers. The central midgut mesoderm, where Dpp and Wg are produced, therefore constitutes a signalling center that organizes the posterior midgut mesoderm.

MATERIALS AND METHODS

Visceral mesoderm (VM) nuclei counting
The enhancer trap PS449, generated in the Scott laboratory, was used to mark VM nuclei. For each measurement, 10 or more stage 14 to 16 embryos (Campos-Ortega and Hartenstein, 1985) were examined under a Zeiss Axioshot equipped with a video camera. Marked nuclei within one of the lateral VM stripe were counted on the scope, with every tenth nucleus marked on the video monitor as a reference point. Measurements of cell numbers in two or more VM stripes from the same embryos revealed variations less than two cells per chamber and are assumed to represent the resolution of the counting process. The posterior boundary of the fourth chamber was defined as the location where the dorsoventral axis of the gut tube becomes oriented perpendicular to the anterior-posterior axis of the embryo.

Embryo histochemistry
Antibody staining and in situ hybridization were done as described in Bilder and Scott (unpublished data). Mutant embryos were identified by lack of various lacZ-marked balancer chromosomes, or, for Df(3R)H999 embryos, the head involution defective phenotype. To make pntP1- and pntP2-specific riboprobes, the plasmids PTrpntP1 and PTrpntP2 were utilized in a PCR reaction with digoxigenin-labelled dUTP using the following primers (5'→3'):
P1: CTCTGCGTTTTTATGTAATGC;
P1 return: GTTGTAAGCTGAACTAAGGAA;
P2: GAATGGCGATTGTAAGA;
P2 return: CATGTGCGAGTGGGCGTGG.

Fly stocks
Lethal excision lines were generated from l(3)7825 following standard methods. pnt88 and UAS-pnt stocks were provided by C. Klümpt, pnt85 and Pmp87 stocks were provided by E. O'Neill. pnt82 and S. rho, spi, aos stocks were obtained from the Bloomington stock center. wg8A14 temperature shifts were done as described in Mathies et al. (1994). The DER allele flb is provided by B. Shilo. DN-DER flies, which carry a dominant negative version of DER, were provided by A. Michelson, who also provided UAS-abdA and UAS-Ubx stocks. Ectopic mesodermal expression was driven by the 24B GAL4-producing line (Brand and Perrimon, 1993).

RESULTS

In a screen of enhancer trap lines expressed in the VM, we found an enhancer trap, l(3)7825, that produces β-galactosidase throughout the VM of the third midgut chamber. l(3)7825 maps to 94F1-2 on polytene chromosomes, the location of the pointed (pnt) gene (Klümpt, 1993). l(3)7825 and imprecise excision alleles derived from it fail to complement pnt mutations (O’Neill et al., 1994 and data not shown). The genetically null mutations pnt2 and pnt88 (Scholz et al., 1993) cause defective midgut development. In the studies that follow, we refer to embryos homozygous for these mutations as pnt mutants.

Midgut expression of pnt
We find that pnt itself, like the l(3)7825 enhancer trap, is expressed in a restricted subset of VM cells. The pnt locus encodes two transcripts, called pntP1 and pntP2 (Klümpt, 1993). These two transcripts contain different 5' exons, but share common 3' exons that encode an ETS DNA-binding domain. Probes specific for pntP1- or pntP2-specific exons, used for in situ hybridization, detect identical transcript patterns in the midgut. The strongest signal is produced by an RNA probe recognizing the pnt common region and these results are presented here to describe pnt expression in the midgut.

pnt expression in the midgut VM commences at stage 13, in a single patch in the central midgut (Fig. 1A). This patch is just posterior to the large basophilic cells of the endoderm, which mark the future site of the central constriction. pnt RNA is also found at the boundaries of the midgut with the foregut and hindgut (Fig. 1B), and in the visceral branches of the trachea. The spindle shape of VM cells and their separation into four patches located dorso- and ventrolaterally around the midgut clearly distinguish pnt expression in the VM from pnt expression in the trachea (Fig. 1A). At stage 16, pnt is expressed throughout the VM of the third midgut chamber, in a pattern identical to lacZ expression in embryos carrying l(3)7825. There is a slight but detectable gradient of pnt expression: the cells that border the central constriction display higher levels of pnt than the cells that border the posterior constriction (Fig. 1C). Odd-paired (opa) is produced throughout the VM of the first and fourth midgut chambers (Fig. 1D; Cimbora and Sakonju, 1995). Examination of l(3)7825 embryos stained for β-galactosidase and opa reveals that the boundaries of gene expression abut, and that no cells express both pnt and opa (Fig. 1D).

pnt is regulated by abdA and Ubx
The restricted expression of pnt in the VM suggested that it might be a target of Hox gene regulation. pnt expression in the third midgut chamber occurs in cells that produce the abdA transcription factor. In abdA mutant embryos, the midgut expression of pnt is wholly absent, while pnt transcription in trachea, nerve cord, foregut and hindgut is unchanged (Fig. 2B). The sufficiency of abdA for pnt activation was tested by driving abdA expression throughout the mesoderm, including the VM, using the GAL4-UAS system (Brand and Perrimon, 1993). pnt is activated by ectopic abdA, but only in the second midgut chamber and in the budding gastric caeca (Fig. 2C). The resultant pattern of pnt is complementary to the sites of
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opa expression in wild type. Although opa is also a target of abdA regulation (Cimbora and Sakonju, 1995), opa expression is unchanged in ectopic-AbdA embryos (data not shown).

Ubx expression at the central constriction flanks the anterior boundary of pnt expression. Interpreting the effects of Ubx mutations on pnt is difficult because abdA, dpp and wg transcription are all affected. Instead, we examined pnt RNA in embryos in which Ubx was expressed throughout the mesoderm. These embryos do not produce pnt RNA in the midgut VM and display the smaller third midgut chamber expected from loss of pnt function (Fig. 2D, see also Fig. 4). The anterior boundary of pnt expression, at the site of the central constriction, is thus apparently set by both abdA activation and Ubx repression.

wg signaling determines the extent of pnt activation by abdA

The previous observations raise the question of why abdA, which is produced throughout the third and fourth chambers, activates pnt only in the third chamber and opa only in the fourth. opa does not expand in pnt mutants (Fig. 5H) and pnt does not expand in opa mutants (data not shown). Ectopically expressed opa has no effect on pnt expression and ectopically expressed pntP1 or pntP2, or a constitutively active pnt (pnt-VP16) (Klaes et al., 1994) has no effect on opa expression (data not shown). Thus, the complementary expression patterns of pnt and opa do not result from mutual repression and must be regulated by other factors.

One candidate regulator of pnt expression is wg. In the VM, wg is expressed just posterior to the central constriction, overlapping pnt expression (Fig. 3B). The range of activity of secreted Wg might therefore determine the extent of pnt activation within the abdA domain. wg, like pnt and opa, is activated by abdA and exd (Rauskolb and Wiechaus, 1994 and

Fig. 1. pnt expression in the gut. (A) pnt riboprobe staining in a wild-type stage 13 embryo. pnt is transcribed in a narrow patch of visceral mesoderm cells in the posterior midgut. pnt is also transcribed in the visceral branch of the trachea. (B) A wild-type stage 13 embryo stained for pnt and Con. In addition to its midgut expression, pnt is found at the sites of junction of the midgut with the foregut (fg) and hindgut (hg). (C) At stage 16, pnt RNA is found throughout the third midgut chamber, with a slight anterior-posterior gradient of levels. (D) Embryos carrying the pnt enhancer trap l(3)7825 stained for β-galactosidase and opa. pnt and opa are continuous but do not overlap; the interface of the expression domains is the site of the posterior constriction (D).

Fig. 2. pnt is regulated by Hox genes. (A) Wild-type embryos produce pnt transcript in the third midgut chamber (arrowheads). (B) pnt RNA is absent from the VM of abdA embryos; note the absent posterior constriction of abdA embryos. (C) In embryos with ectopic abdA expression, pnt is activated ectopically in the second chamber and in the gastric caeca (arrows). (D) Ectopic expression of Ubx represses pnt ; note the small third chamber expected from loss of pnt activity (cf. Fig. 4B).
A requirement for wg in midgut pnt regulation was demonstrated using mutants carrying a temperature-sensitive allele of wg. This allele was used to provide early wg function, allowing segmentation in the ectoderm to take place, before removing its function to assess its later role in midgut development. In wg mutant embryos, pnt is absent from the VM (Fig. 3D). Embryos lacking mid gut expression of dpp exhibit reduced pnt transcription, which does not extend posterior to Con patch 7 (F, compare to wild type in E). Ectopic wg leads to an increase in the levels and posterior extent of pnt (G) production, while ectopic dpp does not result in a significant expansion of pnt (H). In wild-type embryos, opa expression is complementary to that of pnt, sharing a border at Con patch 8; note the boundaries of anterior opa between Con patches 3 and 5 (I). In dpp mutant embryos, posterior opa expands anterior to Con patch 8, while anterior opa expands anterior to Con patch 3 and posterior to Con patch 5 (J). Ectopic Dpp supresses opa throughout the midgut VM (K).

**Data not shown.**

**dpp signaling restricts opa activation by abdA to the fourth midgut chamber**

dpp, an additional candidate regulator of midgut pnt expression, collaborates with wg in regulating a number of genes, including tsh in the midgut (Mathies et al., 1994). In dpp mutant embryos, pnt transcript is present but in a domain much reduced as compared to wild type. To carefully document changes of gene expression in dpp mutant embryos, we made...
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use of connectin (con) expression as a landmark for cell positions along the VM and ectoderm. Con is produced in 11 patches along the midgut VM from stage 11 to stage 14; these patches align with ectodermal engrailed (en) stripes and reveal metameric pattern in the VM (Gould and White, 1992; Bilder and Scott, unpublished data). In stage 13 wild-type embryos, pnt expression overlaps Con patch 7 and extends to the anterior boundary of patch 8 (Figs 3E, 5C). opa RNA overlaps Con patch 8 and extends posterior to Con patch 11 (Figs 3L, 5D), again documenting the complementary expression of pnt and opa. In stage 13 dpp mutant embryos, the anterior boundary of pnt expression is unaffected, but little pnt transcript is visible posterior to Con patch 7 (Fig. 3F). Thus pnt expression is reduced in its posterior extent in dpp mutants.

Further data suggest that the reduced pnt expression observed in dpp mutants is the result of reduced wg production in these embryos. Embryos with dpp ectopically expressed throughout the VM have only a slight stimulation of pnt expression (Fig. 3H) and the posterior boundary does not change significantly. Furthermore, embryos in which both dpp and wg are expressed simultaneously throughout the VM do not have increased pnt transcription as compared to ectopic-Wg embryos (data not shown). Therefore dpp regulates pnt mainly or entirely through its effects on wg.

dpp is, however, required for patterning the posterior midgut in a process that is not dependent on wg. In dpp mutant embryos, a dramatic derepression of opa occurs in the VM (Fig. 3J; Cimbora and Sakonju, 1995). By stage 13, opa RNA has expanded both posterior to its wild-type anterior border at Con patch 6 and anterior to its wild-type posterior border at Con patch 7. The complementary expression of pnt and opa is preserved. Correspondingly, ectopic dpp represses opa, completely in the anterior and to a great extent in the posterior (Fig. 3K). In summary, wg is required for pnt activation in the third midgut chamber while dpp independently represses opa in this region, restricting opa to the fourth chamber.

pnt is required for morphogenesis of the posterior midgut

pnt mutant embryos have striking defects in morphogenesis of the posterior midgut. Instead of four chambers containing

Fig. 4. pnt is required for placement of the posterior constriction. Stage 16 embryos stained with anti-MHC antibody. (A) In wild-type embryos, the four midgut chambers (1-4), divided by the anterior, central and posterior midgut constrictions (A, C, P) are of approximately equal size. (B) In pnt mutant embryos, the third chamber is drastically reduced in size. Incomplete formation of the anterior constriction and gastric caeca is also seen.

Fig. 5. pnt mutants do not have altered expression of midgut patterning genes. (A,B) pnt embryos activate tsh transcription normally (arrows in B, compare WT in A) at the sites of the anterior and central constrictions, reflecting the wild-type expression of VM patterning genes in the anterior and central midgut. (C-H) pnt (C,E,G) or opa (D,F,H) RNA in embryos also stained for Con. (C-F) Stage 13; (G,H) stage 16. (C) In wild-type embryos, pnt is found from the anterior margin of Con patch 7 to the anterior margin of Con patch 8. (D) opa is found from the anterior margin of Con patch 8 to the posterior margin of Con patch 7. opa is also found in the anterior midgut, from the posterior margin of Con patch 3 to the anterior margin of Con patch 5. (E,F) In pnt2 mutants, which still produce pnt transcript, transcription of both pnt (E) and opa (F) in relation to Con patches is unchanged. (G,H) Following constriction formation, pnt is found throughout the much smaller third chamber (G, compare Fig. 1C), while opa is found throughout the fourth chamber (H; compare Fig. 1D).
roughly equal volumes of yolk, as is seen in stage 16 wild-type embryos (Fig. 4A). \textit{pnt} mutant embryos have a third midgut chamber that is greatly reduced in size (Fig. 4B). The central and posterior constrictions that bound this chamber form normally but are located much more closely together than in the wild-type gut. This phenotype is fully penetrant. In addition, 85% of \textit{pnt} embryos fail to form the anterior constriction completely: the constriction initiates at its proper site but does not significantly constrict the midgut. No elongation of gastric caeca is seen in these embryos (data not shown).

To determine which of the two co-expressed \textit{pnt} transcripts is required for midgut morphogenesis, we examined the midgut phenotypes of embryos homozygous for \textit{pnt} mutations that delete either \textit{pntP1} or \textit{pntP2}-specific exons (O’Neill et al., 1994). Embryos homozygous for the \textit{pntP1} mutation \textit{pnt}533 or the \textit{pntP2} mutation \textit{pnt}788, or transheterozygous for these mutations and \textit{pnt}688, have wild-type midgut development. The \textit{pnt} transcripts are therefore functionally redundant for posterior midgut morphogenesis.

\textbf{Midgut patterning genes are normally expressed in \textit{pnt} mutants}

The small third chamber observed in \textit{pnt} embryos could result from a shift in expression of VM patterning genes required for placement of the central or posterior constrictions along the midgut axis. Alternatively, loss of \textit{pnt} could lead to a decrease in the number of VM cells in the third chamber without changing regulating gene expression.

An analysis of midgut patterning gene expression indicates that cell fates in the midgut VM are properly specified in these embryos. In \textit{pnt} embryos, \textit{tsh} expression is found in wild-type patterns at the sites of the central and partially formed anterior constrictions (Fig. 5B). \textit{tsh} expression in the anterior VM requires \textit{Antp}; in the central VM, it requires \textit{Ubx}, \textit{abdA}, \textit{dpp} and \textit{wg} (Mathies et al., 1994). All four regulators are properly expressed in \textit{pnt} embryos (data not shown), indicating that the anterior and central constrictions are appropriately placed in \textit{pnt} mutants.

Posterior midgut cell fate markers are also normally expressed in \textit{pnt} mutants. In \textit{pnt\textsuperscript{2}} mutant embryos, which produce non-functional \textit{pnt} transcript, \textit{pnt} expression at stage 13 is wild type, covering Con patch 7 and extending to the anterior boundary of Con patch 8 (Fig. 5E). Con patches 7 and 8 align appropriately with ectodermal \textit{en} stripes in these embryos (data not shown). \textit{opa} expression in \textit{pnt} embryos is also normal, with no expansion anterior to Con patch 8 (Fig. 5F). At stage 16 when the posterior constriction appears as a landmark, it is clear that the third midgut chamber, although smaller than normal, is composed exclusively of \textit{pnt}-expressing cells as in wild type. The fourth chamber is composed exclusively of \textit{opa}-expressing cells, also as in wild type (Fig. 5G,H). Thus, \textit{pnt} mutations do not cause changes in expression of known midgut patterning genes in relation to either early (Con patches) or late (constriction) midgut landmarks.

\textbf{\textit{pnt} mutants have an insufficient numbers of third chamber VM cells}

Early \textit{pnt} embryos have wild-type anterior-posterior information along the midgut axis, so the small third chamber of \textit{pnt} mutants must arise from a spatially restricted reduction in VM cell number. Such a defect would change the apparent location of the posterior constriction, while not changing gene expression outside the affected domain. To count VM cell numbers in the midgut, we used an enhancer trap, P5449, which produces nuclear-localized \(\beta\)-galactosidase exclusively in the midgut VM. At stage 16, VM nuclei are aligned in four single files located at dorso- and ventrolateral locations on each side of the midgut (Fig. 6A; Reuter and Scott, 1990). Using P5449, the sizes of midgut chambers were measured by counting VM nuclei along a single file between the constrictions. This number is highly replicable (Fig. 6C). In \textit{pnt} embryos, the third chamber is dramatically reduced, containing only 21 VM nuclei as compared to 36 in wild-type embryos (Fig. 6B). The first, second and fourth midgut chambers of \textit{pnt} embryos contain normal numbers of cells. The reduced size of the third midgut chamber is thus due primarily to a reduction of VM cell number in the third chamber. Neighboring chambers do not appear to expand at the expense of the third.

The reduction in VM cell number in \textit{pnt} mutants could take
place by any of three mechanisms: apoptotic elimination of VM cells destined to fill the third chamber, insufficient generation of third chamber cells, or transformation of third chamber cells to a non-VM fate. The first possibility was tested by examining pnt mutants in a genetic background [Df(3R)H99] that prevents programmed cell death in the embryo (White et al., 1994). pnt Df(3R)H99 double mutants have the small third chamber and incomplete anterior constriction of pnt mutants (Fig. 7). Acridine orange staining (Abrams et al., 1993) was also used to demonstrate the absence of apoptosis in pnt mutant midguts (data not shown), suggesting that inappropriate cell death is not the cause of reduced VM cell numbers in pnt mutants. The second possibility was tested by examining cell divisions in the VM with an antibody that detects mitotic chromosomes (anti-phospho Histone H3, de Nooij et al., 1996). In agreement with previous studies (Bate, 1993), we find that all VM mitoses are complete by stage 13, prior to pnt expression. Therefore pnt is not required for cell division in the VM. Because defects in cell death or cell proliferation do not occur in the VM of pnt mutants, the loss of third chamber VM cells must be due to a transformation of cell fate to a cell type that does not express P5449 and does not contribute to the anteroposterior size of the third chamber.

P5449-marked embryos also reveal defects in VM cell organization throughout the length of the midgut of pnt mutants, in addition to the underproduction of third chamber cells discussed above. In wild-type embryos, VM cells undergo a mitosis at stage 12 that is oriented in the dorsal-ventral axis and neatly splits the VM into two rows of cells on either side of the midgut (Fig. 8A). The VM cells maintain their alignment as they migrate to their final positions in rows located dorso- and ventrolaterally on the gut (Fig. 8B). Stage 13 pnt embryos have defects in separation of the VM cells into distinct files (Fig. 8C); the most severely affected cells lie in the central VM. By stage 16, VM nuclei are often scattered around the surface of the midgut, in contrast to the orderly files seen in wild type (Fig. 8D). pnt activity is thus required for the concerted dorsoventral movement of VM cells.

**DISCUSSION**

The morphology of the midgut, reflected in the placement of the constrictions and the size of the midgut chambers, is a sensitive indicator of anterior-posterior patterning in the VM. Division of the midgut chambers is highly replicable, both in time (Broadie et al., 1992) and in space (Fig. 6). Spatial replicability is reflected in the precise allocation of VM cell numbers to the four midgut chambers. The present work demonstrates that pnt is a Hox-activated regulator required to produce the proper number of VM cells in the third midgut chamber. Regulation of pnt and opa by a combination of Hox proteins and diffusible signals divides the posterior midgut into distinct domains that demarcate the third and fourth midgut chambers. Other domains of Hox action may be subdivided using similar regulatory processes.

**A model for posterior midgut morphogenesis: differential activation of abdA target genes**

We suggest the following model for morphogenesis of the posterior midgut (Fig. 9). abdA activates three targets in distinct subsets of its broad domain of expression: wg at the anterior boundary of Con patch 7, pnt from anterior Con patch 7 to anterior Con patch 8 and opa from anterior Con patch 8 through Con patch 11. Dpp signaling plays a central role in setting these distinct expression domains. The initial activation of wg by abdA requires dpp. opa is activated in all abdA-expressing cells that do not receive a Dpp signal, defining the site of the posterior constriction. Finally, wg, in collaboration

![Fig. 7. The pnt midgut phenotype is not suppressed by blocking apoptosis. (A) Df(3R)H99 embryos, which undergo programmed cell death, show wild-type midgut morphogenesis. Embryos homozygous for both pnt and Df(3R)H99 show the reduced third midgut chamber and partially formed anterior constriction characteristic of pnt mutants (B), suggesting that the pnt midgut phenotype does not result from apoptosis.](image)

![Fig. 8. VM disorganization throughout the midgut of pnt embryos. (A,B) WT and (C,D) pnt embryos carrying P5449 stained with anti-β-galactosidase. Segregation of the two files of VM cells following the fourth post-blastoderm mitosis at stage 12 occurs in WT embryos evenly along the length of the midgut (A), leading to alignment of the VM cells in a single file along the midgut at stage 16 (B). In pnt embryos, defects in VM cell segregation are evident immediately after the stage 12 mitosis, producing a junction in the central midgut (C). This leads to distribution of the VM cells around the circumference of the stage 16 pnt midgut (D); cells are most widely distributed in the first chamber (compare to the fourth chamber, just out of focus).](image)
with \textit{abdA}, activates \textit{pnt} to generate the appropriate number of cells in the third midgut chamber, positioning the posterior constriction at the proper distance from the central constriction and partitioning the posterior midgut appropriately. Fine patterning of the posterior midgut is achieved by the activity of diffusible signals emanating from the central midgut, a remarkably long-range organizing effect.

\textbf{Regulation of \textit{pnt}, a target of \textit{abdA}}

The subdivision of the posterior midgut into the third and fourth chambers is reflected molecularly in the complementary expression patterns of the genes \textit{pnt} and \textit{opa}. \textit{pnt}, like other genes required for midgut patterning, is regulated by Hox genes. \textit{abdA} is required for \textit{pnt} expression and, in ectopic-\textit{AbdA} embryos, ectopic \textit{pnt} is induced. Since ectopic Ubx represses \textit{pnt} while having no effect on other \textit{abdA} targets, the anterior boundary of \textit{pnt} expression is determined both by \textit{abdA} activation and by \textit{Ubx} repression. \textit{pnt} expression in the VM is first detectable at stage 13, significantly later than other Hox targets whose expression is evident from the initial formation of the VM at stage 11. In other embryonic and adult tissues studied, \textit{pnt} function is regulated by receptor tyrosine kinase signalling mediated by spitz group genes. However, spitz group mutants have wild-type midgut development (data not shown), so the regulation of \textit{pnt} in the VM is quite distinct from elsewhere.

\textbf{Long-range modulation of \textit{abdA} action by \textit{wg} and \textit{dpp}}

The first known step in AP patterning of the midgut is the non-overlapping differential expression of homeotic (Hox) genes. The repression of \textit{Ubx} by \textit{abdA} guarantees that these domains will be complementary. This in turn leads to non-overlapping, adjacent, expression of the Hox targets \textit{dpp} and \textit{wg}. The complementary domains of expression of \textit{pnt} and \textit{opa}, seen both in wild type and in mutant and ectopic expression contexts, are shown in this work to be regulated by the diffusible signals Dpp and Wg.

Although \textit{pnt} transcription requires \textit{abdA}, \textit{pnt} is transcribed only in a subset of the \textit{abdA}-expressing VM cells. We have provided evidence that \textit{wg} plays a critical role in restricting \textit{pnt} expression. The loss of \textit{pnt} expression in the absence of \textit{wg} or \textit{abdA} indicates the need of both genes for \textit{pnt} transcription. The extent of \textit{pnt} ectopic activation following ubiquitous \textit{wg} and \textit{abdA} expression strongly suggests that the two proteins are required simultaneously for \textit{pnt} activation. Thus signalling by \textit{Wg} determines the extent of \textit{pnt} activation by \textit{abdA}, providing a molecular explanation for the spatially limited action of \textit{abdA} on \textit{pnt}.

The nuclear effectors of the Wg signaling pathway have recently been identified in \textit{Drosophila} and vertebrates (Brunner et al. 1997; Behrens et al., 1996; Huber et al., 1996; Molenaar et al., 1996). The \textit{Pan/Left} genes encode proteins from the sequence-specific HMG family and have been proposed to act in a complex with the Arm/\beta-catenin protein to stimulate transcription. Lef1 proteins have been proposed to have an architectural function in the regulation of transcription (Grosschedl et al., 1994). By bending DNA they affect the arrangement and actions of other transcription factors. The binding and subsequent bending of \textit{abdA}-regulated enhancers by the Pan/Arm complex might be a prerequisite for \textit{pnt} transcriptional activation by AbdA. Repression of \textit{opa} by \textit{dpp} in the third compartment may take place in a manner analogous to the activation of \textit{pnt} in the third compartment by \textit{wg}; by modulating the ability of \textit{abdA} to activate appropriate targets. The differential readout of the two signals at the level of the \textit{pnt} and \textit{opa} promoter or enhancer region eventually leads to distinct regulatory effects of the same Hox transcription factor.

To regulate posterior midgut cell fates, Dpp and Wg signals move from the central midgut position to spread into the third chamber. Dpp and Wg effects are felt at least 30 cells away from their sources. The central constriction region where \textit{dpp} and \textit{wg} are expressed therefore constitutes an ‘organizer’ that patterns the posterior midgut. This role of Dpp and Wg can be added to two previous functions: activating \textit{tsh} in the mesoderm to form the central constriction and determining endoderm cell fates near the central constriction.

\textit{dpp} and \textit{wg} act in combination in a number of tissues; both independent and interdependent stimulation of target genes have been suggested (Tremml and Bienz, 1992; Campbell et al., 1993; Cohen, 1990; Thüringer and Bienz, 1993; Mathies et al., 1994). Our evidence suggests that \textit{dpp} does not regulate \textit{pnt} independently of \textit{wg}. Although \textit{dpp} is required for proper \textit{pnt} expression and ectopic \textit{dpp} causes increased expression of \textit{pnt}, \textit{dpp} has these same regulatory relationships with \textit{wg} (Immerglück et al., 1990; Reuter and Scott, 1990; Staehling-Hampton and Hoffman, 1994).
limited induction of wg by dpp correlates with the comparatively limited increase of pnt seen in ectopic-Dpp embryos as compared to that seen in ectopic-Wg embryos. Furthermore, simultaneous production of dpp and wg throughout the midgut does not cause pnt to be expressed throughout the abdA domain. Therefore the effects of dpp are probably a result of its effects on wg.

**pnt regulates VM cell number in the third midgut chamber**

In the absence of pnt function, the third midgut chamber is greatly reduced in size. Careful analysis of cell division and cell death in pnt embryos has shown that the reduction in cell number in pnt mutants is not due to apoptosis or to initial underproduction of VM cells. The Con stripes in the VM are properly spaced at stage 13 in pnt mutants and patterning gene expression is normal. The first visible midgut defects in pnt mutants are seen at stage 13, when dorsoventral migration of the VM cells is aberrant. Only 3 hours later, the third chamber VM is about half of its normal size, while the anterior and central constrictions are appropriately placed. If a shift in the posterior constriction was occurring, an increased number of cells should be seen in the fourth chamber. We have not seen such an increase, although the difficulty in replicably ascertaining the posterior limit of the fourth chamber prevents us from completely excluding an increase in cell numbers in the fourth chamber. As a more likely alternative, we propose that the absence of pnt from third chamber VM cells at stage 13 causes many of them to undergo a transformation to a non-VM cell fate in which they do not express P5449 and do not contribute to the length of the midgut.

The defective formation of anterior constrictions and gastric caeca seen in many pnt embryos is surprising, since pnt is not expressed in the VM at these sites and pnt mutations do not change the expression of genes known to be required for formation of these structures. It is possible that the reduction of VM cell numbers in pnt mutants itself leads to structural disorganization, as an overall smaller VM attempts to encase and constrict a wild-type-sized midgut endoderm. In wild-type embryos, constrictions seem to form by a ‘drawstring’ mechanism, in which simultaneous contraction of cytoskeletal elements within VM cells, evenly distributed around the circumference of the midgut at a specific site on the AP axis, leads to a pinching of VM into the yolk (Reuter and Scott, 1996). Functional interaction of beta-catexin with the transcription factor LEF-1. Nature 382, 638–42.


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