Development 110, 1041–1050 (1990)
Printed in Great Britain © The Company of Biologists Limited 1990

A *Drosophila* growth factor homolog, *decapentaplegic*, regulates homeotic gene expression within and across germ layers during midgut morphogenesis

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Summary

The *decapentaplegic* (*dpp*) gene product, a member of the transforming growth factor-β family, is required in *Drosophila* embryos for normal gastrulation and the establishment of dorsal–ventral polarity in the embryo. *dpp* is also expressed at specific positions in the visceral mesoderm along the developing midgut. We find that mutations that eliminate the visceral mesoderm expression of *dpp* lead to defects in midgut morphogenesis and alter the spatially localized expression of the homeotic genes *Sex combs reduced* (*Scr*), *Ultrabithorax* (*Ubx*), and *Antennapedia* (*Antp*) in the visceral mesoderm. The extracellular *dpp* protein migrates from the visceral mesoderm across the apposing endodermal cell layer in a region of the endoderm that expresses the homeotic gene *labial* (*lab*). Mesodermal expression of *dpp* is required for the expression of *lab* in these endodermal cells indicating that *dpp* mediates an inductive interaction between the two germ layers. We propose that extracellular *dpp* protein regulates gut morphogenesis, in part, by regulating homeotic gene expression in the visceral mesoderm and endoderm of the developing midgut.

Key words: *decapentaplegic*, *Ultrabithorax*, *Sex combs reduced*, *Antennapedia*, *abdominal A*, *labial*, visceral mesoderm, endoderm.

Introduction

The final stages of midgut development in the *Drosophila* embryo involve the migration and stretching of cells in two distinct germ layers to enclose the yolk. The inner endodermal layer is composed of large polyploid cells, while the outer visceral mesoderm layer is composed of small euploid cells. The double-walled gut tube is then compartmentalized into four morphologically distinct chambers and four caeca, long appendages extending from the anterior end of the midgut tube. The four chambers of the midgut are created when three constrictions form at specific positions along the length of the midgut. The caeca are formed as buds off the anterior end of the midgut which elongate and extend into the head regions of the embryo. In the final stages of embryogenesis, the compartmentalized midgut stretches out to form a long convoluted tube (Poulson, 1950; Campos-Ortega and Hartenstein, 1985; Reuter and Scott, 1990). The specific roles in gut function of the four midgut chambers are not known.

Four homeotic genes expressed in the visceral mesoderm of the midgut are crucial for gut morphogenesis (Tremml and Bienz, 1989*a*; Reuter and Scott, 1990). *Scr* is expressed in the visceral mesoderm cells at the anterior end of the midgut immediately posterior to the budding gastric caeca. In the absence of *Scr* expression, the gastric caeca do not develop properly (Reuter and Scott, 1990). Adjacent and posterior to the domain of *Scr* expression is a domain of *Antp* expression. *Antp* is expressed by visceral mesoderm cells surrounding the first constriction and, in the absence of *Antp* expression, the first constriction does not form (Tremml and Bienz, 1989*a*; Reuter and Scott, 1990). Immediately posterior to the *Antp* domain is a domain of *Ubx* expression. *Ubx* is expressed in the visceral mesoderm cells of the anterior half of the second constriction with expression extending anteriorly toward the first constriction. In the absence of

Ubx expression, the second constriction does not form (Bienz and Tremml, 1988; Tremml and Bienz, 1989*a*; Reuter and Scott, 1990). Immediately posterior to the *Ubx* domain, *abdominal A* (*abdA*) is expressed in the visceral mesoderm cells of the posterior midgut. The anterior limit of the *abdA* domain is the posterior half of the second constriction and includes the region around the third constriction. Without *abdA* function, neither the second nor the third constrictions form (Tremml and Bienz, 1989*a*). The mechanisms by which these four homeotic genes influence the formation of the constrictions are not known, nor is it clear whether constriction formation is driven by the mesoderm or formed through the actions of both germlayers. Constriction formation is associated with the elaboration of extensive microtubular arrrays in the visceral mesoderm cells at the site of the constriction (Reuter and Scott, 1990).

The molecular mechanisms regulating the domains of homeotic gene expression in the visceral mesoderm are not known. The expression domains of the four homeotic genes in the visceral mesoderm of the developing midgut are non-overlapping (Tremml and Bienz, 1989*a*). This is in contrast to the expression patterns in the embryonic ectoderm where the *Antp* domain extends posteriorly through those of *Ubx* and *abdA*, and the *Ubx* domain extends posteriorly through that of *abdA* (Carroll *et al.* 1988; Karch *et al.* 1990). The regulatory interactions between the homeotic genes in the visceral mesoderm differ from the regulatory interactions observed between homeotic genes in the ectoderm. For example, *Antp* negatively regulates *Scr* in the ectoderm (Riley *et al.* 1987), but positively regulates *Scr* in the visceral mesoderm (Reuter and Scott, 1990). The pair rule gene *ftz* is required for activation of *Scr*, *Antp*, and *Ubx* expression in the ectoderm (Ingham and Martinez-Arias, 1986) but has little effect on the expression of these genes in the visceral mesoderm (Tremml and Bienz, 1989*b*). It seems likely that the regulation of homeotic gene expression in the visceral mesoderm involves genes other than those used in the ectoderm. Genes that are expressed in specific patterns in the visceral mesoderm are good candidates for genes that may participate in the regulation of homeotic gene expression in the visceral mesoderm.

One gene expressed in specific regions of the developing midgut is the *Drosophila decapentaplegic* (*dpp*) gene, a member of the transforming growth factor-β (TGF-β) family (Padgett *et al.* 1987; Reviewed by Hoffmann, 1990). The visceral mesoderm expression of *dpp* was originally noted in the initial characterization of *dpp*'s embryonic expression (St. Johnston and Gelbart, 1987). During our studies on *dpp* expression in wild-type and mutant embryos using *in situ* hybridization to whole embryos (Tautz and Pfeiffle, 1989), we noted that one domain of *dpp* expression in the midgut visceral mesoderm appears to be coextensive with the reported parasegment 7 domain of *Ubx* expression in the midgut visceral mesoderm (Jackson and Hoffmann, in preparation). Our subsequent investigations are the subject of this report and a companion article (Reuter *et al.* 1990).

dpp is required for normal gastrulation and dorsal–ventral polarity in the *Drosophila* embryo (Irish and Gelbart, 1988). Its patterns of embryonic expression bear little resemblance to the anterior to posterior organization of the major patterns of homeotic gene expression. Rather, the ectodermal expression of *dpp* is regulated along the dorsal to ventral axis of the embryo beginning with a dorsally restricted stripe of *dpp* mRNA at cellular blastoderm (St. Johnston and Gelbart, 1987; Jackson and Hoffmann, in preparation). Midway through embryogenesis, dorsal and lateral stripes of *dpp* mRNA are detected which extend along the anterior–posterior axis through all the thoracic and abdominal segments. *dpp* null mutant embryos exhibit a transformation of dorsal and lateral hypoderm to ventral hypoderm, the most completely ventralized phenotype observed to date in the *Drosophila* embryo. Mutations that affect the 5′ regulatory sequences, a region of five alternate *dpp* promoters called the shortvein region, give rise to embryos with normal dorsal–ventral polarity, but the animals die as larvae with some visible defects in internal organs (Segal and Gelbart, 1985). *dpp* mutations affecting 3′ regulatory sequences, called the disk region, undergo normal embryogenesis and larval development but display an array of defects in the adult cuticular structures derived from the larval imaginal disks (Spencer *et al.* 1982; St. Johnston *et al.* 1990; Masucci *et al.* 1990).

Biochemical analysis of *dpp* protein produced in cultured *Drosophila* cells has indicated that *dpp*, like the other members of the TGF-β family, forms a dimer that is proteolytically processed and secreted from cells (Panganiban *et al.* 1990). Members of the TGF-β family have been shown to exert a wide variety of effects on cell proliferation, cell differentiation, extracellular matrix elaboration and hormone secretion (Roberts and Sporn, 1988). The effects of any specific factor in the family can vary depending on the target tissue and the dose. While the importance of TGF-β to wound healing has been well established, the roles of these factors in development are less well characterized. Several of the factors are expressed in a tissue specific fashion in developing mouse skin and bone, leading to the hypothesis that members of the TGF-β family establish regulatory cascades of interactions during sequential steps in the development of these tissues (Lyons *et al.* 1989*a*). Probably the most pertinent observation about the developmental role of a member of the TGF-β family is the regulation of the *Xenopus* homeotic gene *xhox-3* by a *Xenopus* TGF-β homolog, XTC-MIF (Ruiz i Altaba and Melton, 1989). XTC-MIF induces animal cap cells to become mesoderm and induces *xhox-3* expression to a level that determines an anterior mesodermal fate. A different growth factor, fibroblast growth factor, also induces mesoderm, but establishes higher levels of *xhox-3* expression which gives rise to posterior mesoderm. Therefore the presence of a growth factor can specify the positional fate of cells through the regulation of homeotic genes.

We find that a *Drosophila* member of the TGF-β family, *dpp*, also regulates homeotic gene expression. *dpp* negatively regulates the homeotic gene *Scr* and positively regulates the homeotic gene *Ubx* in the visceral mesoderm cells of the midgut. Limited migration of *dpp* protein from the visceral mesoderm to the endoderm is required to induce expression of a third homeotic gene, *labial* (*lab*), in the endodermal cells. The expression of *dpp* in the visceral mesoderm is required for proper gut morphogenesis, specifically for the formation of the gastric caeca and the second midgut constriction. These same two features of gut morphogenesis require expression of *Scr* and *Ubx*, respectively.

Materials and methods

The *Antp*, *Ubx* and *Scr* mutant embryos used in these experiments were generated as described by Reuter and Scott (1990). *abdA* mutant embryos were generated by homozygosing the $abdA^{M1}$ allele (Sanchez-Herrero *et al.* 1985). The dpp^{shv} alleles used in these experiments were isolated by Segal and Gelbart (1985). The molecular lesions associated with these mutations are summarized in Fig. 1C and were described by St. Johnston *et al.* (1990). To generate mutant embryos, balanced stocks of the various dpp^{shv} alleles were outcrossed to wild-type Canton S females. dpp^{shv}/Canton S virgins were then mated similarly outcrossed males carrying a different dpp^{shv} allele. The embryos from these crosses were fixed and stained as described below. To avoid the possibilities that any defects observed were due to mutations other than *dpp* or to the genetic background in which a given dpp^{shv} allele had been generated, each cross was performed with two dpp^{shv} different alleles which had been induced on unrelated starting chromosomes.

Scr, *Ubx* and *Antp* antibodies used are described in Reuter and Scott (1990); *lab* antibodies are described in Diederich *et al.* (1989). Antibodies to *dpp* were generated as described by Panganiban *et al.* (1990). To affinity purify the resulting polyclonal antibodies, sera were first passed over Affigel-protein A columns (BioRad) to purify the IgG. Sera were diluted with an equal volume of 1.5 M glycine; 3 M NaCl pH 8.9 prior to loading on the columns. The columns were washed with 5 volumes of this buffer prior to elution with 0.1 M glycine; 20 mM NaCl pH 3.2. IgG containing fractions were cut with 50 % (final concentration) saturated ammonium sulfate, and centrifuged at 10000 *g* to pellet the precipitate. The IgG was then dialyzed against PBS (10 mM KPO_4; 140 mM NaCl pH 7.2) prior to loading on a *dpp* affinity column. *dpp* affinity columns were generated by coupling 5–10 mg of SDS–PAG purified and electroeluted pG17 inclusion bodies (described in Panganiban *et al.* 1990) in 0.1 M MOPS pH 7.5 to Affigel 10 (BioRad) according to manufacturers instructions. IgG fractions containing antibodies to *dpp* were loaded on the affinity columns at 5–10 ml h^{-1}. The columns were washed extensively with 10 mM $NaPO_4$ pH 7.0 to reduce the buffer concentration prior to elution with 0.1 M glycine pH 2.5. 1 ml fractions were collected directly into 100 μl of 1 M $NaPO_4$ pH 8 to neutralize the glycine (Harlow and Lane, 1988). Antibody concentration was determined by absorbance at 280 nm (A280 of 1=0.56 mg ml^{-1}). BSA (Sigma) was added to a final concentration of 1 mg ml^{-1} and sodium azide was added to 0.02 % prior to storage at 4 or −20°C.

Embryos for immunohistochemistry were dechorionated, fixed and devitellinized using a modification of the methods of Mitchison and Sedat (1983). Briefly, embryos were dechorionated in 50 % bleach for two minutes, rinsed with distilled water and fixed for 20 min in a two phase system composed of 1 part heptane and 1 part 4 % paraformaldehyde in PBS (PF-PBS). The PF-PBS was then replaced with methanol, leaving the embryos at the interface. To devitellinize the embryos, the tubes were shaken gently until the majority of the embryos had settled through the methanol. The heptane and methanol were then removed with a pipette. Embryos were rinsed 3 times with absolute ethanol and treated with 3 % hydrogen peroxide (in ethanol) for 1 min to inactivate endogenous peroxidases. For optimum staining with the *dpp* antibody, it was necessary to stain the embryos immediately following fixation. All other antibodies gave excellent staining with embryos fixed 3–6 months previously and stored at −20°C in ethanol. Antibody incubations and washes were modified from the methods of Carroll and Scott (1985). Embryos were rehydrated through a series of ethanol: PT (PBS plus 0.1 % Triton X-100) 50:50; 25:75; 0:100 and blocked at least 3 h in PBT (PBS plus 0.2 % BSA plus 0.1 % Triton X-100) with 2 % normal goat serum (Sigma). All subsequent washes and incubations were in PBT unless otherwise specified.

Primary antibodies were incubated with embryos overnight at 4°C. The *dpp* antibody was used at 1 μg ml^{-1}; *lab* was used at 1:150; and *Scr*, *Antp* and *Ubx* monoclonals were used at 1:3, 1:5 and 1:5, respectively. At least 10 1 ml washes were carried out over 4–8 h after each incubation with antibody or with streptavidin–HRP. Secondary antibodies, biotinylated goat anti-rabbit IgG (Vector Laboratories) or HRP-conjugated goat anti-mouse IgG (BioRad), were preadsorbed against embryos and incubated with the embryos for 2 h at room temperature. Final dilutions in PBT plus 2 % NGS were 1:600 for the anti-rabbit and 1:400 for the anti-mouse. Washes were as described above. Embryos probed with the monoclonals were then developed in PBT; 0.25 mg ml^{-1} diaminobenzidine (Sigma); and 0.03 % hydrogen peroxide (Sigma). Embryos probed with the *lab* and *dpp* antibodies were incubated for 1 h at room temperature with a 1:300 dilution of streptavidin-HRP (BRL) which had been preadsorbed against embryos. Embryos were then washed and developed as described above. Stained embryos were washed extensively with PBT and PBS, dehydrated through an ethanol series and cleared in methyl salicylate (Sigma). The embryos were then either mounted in salicylate or embedded in Epon 812–Araldite prior to observation by differential interference contrast optics on a Zeiss Axiophot. 6 μm sections were cut from embedded embryos using a Reichert-Jung Ultracut E.

Embryos for RNA *in situs* were fixed in a formaldehyde and probed with a digoxigenin labelled *dpp* cDNA probe according to the procedures of Tautz and Pfeiffle (1989). Hybridized RNA was visualized *via* use of an alkaline phosphatase conjugated antibody to digoxigenin using the Genius kit from Boehringer Mannheim Pharmaceuticals.

Results

dpp *is expressed at specific locations along the developing gut*

dpp RNA is expressed in six positions along the developing larval gut (Fig. 1A). *dpp* expression is first detected in the midgut visceral mesoderm at the beginning of germband shortening, stage 12 (Campos-Ortega and Hartenstein, 1985). By the time germband shortening is complete, six specific regions of *dpp* expression are detected along the gut tube. These

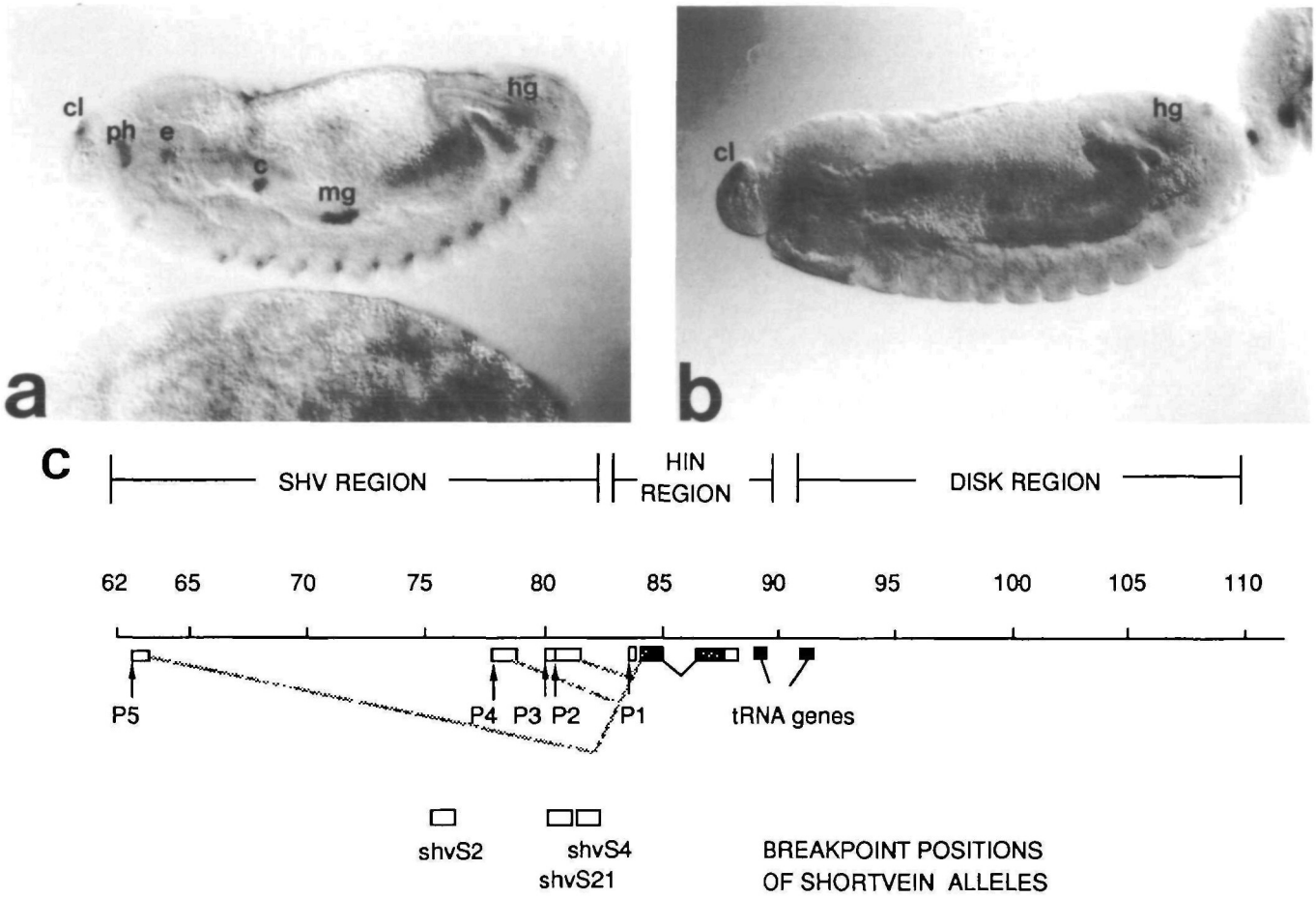

Fig. 1. *dpp* RNA expression in stage 14 wild-type (A) and dpp^{S21}/dpp^{S4} (B) embryos. Lateral views with anterior left and dorsal up photographed at 20×. The wild-type embryo expresses *dpp* in the clypeolabrum (cl), the hindgut (hg), and in the pharynx (ph) and the esophagus (e) of the foregut and the gastric caeca (c) at the anterior end of the midgut and at the anlage of the second midgut constriction (mg). The dpp^{shv} mutant embryo lacks expression in the midgut and the foregut expression is greatly reduced. Panel C contains a map (modified from St. Johnston *et al.* 1990) of the *dpp* gene with the breakpoints of the dpp^{shv} mutants used in these experiments indicated. Note that dpp^{shv} mutations map within the transcribed region of the gene, but are 5′ to the coding exons which are in the Hin (haploinsufficient) region of the gene.

include expression in the ectoderm of the foregut and hindgut and in the visceral mesoderm of the midgut. The two spots of staining in the foregut ectoderm are within the anlage of the pharynx and the esophagus. The two principle regions expressing *dpp* RNA in the midgut are within the anlage of the gastric caeca and the site of the second midgut constriction. Low levels of *dpp* expression can also be detected at the site of the third midgut constriction although this expression is not visible in Fig. 1. This latter expression is under the control of different cis-regulatory elements than the elements required for the other two parts of *dpp* expression in the midgut (Jackson and Hoffmann, in preparation) and is not dealt with further in the present report. In addition, *dpp* is expressed by the ectoderm of the hindgut. The sites of *dpp* expression in the midgut correspond to regions that undergo morphological changes during development. For example, the caeca evaginate from the anterior end of the midgut and extend anteriorly well into the head. *dpp* expression in the gastric caeca is no longer detected when the caeca reach full extension at stage 17. The second midgut constriction occurs in the domain expressing *dpp* in the middle of the midgut. Double labelling experiments using antibodies to *dpp* and *Ubx* indicate that the *dpp* domain overlaps that of *Ubx* (Reuter *et al.* 1990). The domain expressing *Ubx* has been shown to correspond to a metameric unit of the embryo known as parasegment 7 (PS 7) (Tremml and Bienz, 1989*a*). *dpp* expression in PS 7 is maintained during the process of forming the gut tube and then declines. To determine whether *dpp* expression is required for the morphological events of midgut development, we examined gut morphogenesis in *dpp* mutant embryos.

Mutations in the dpp *shortvein region eliminate* dpp *expression in the gut*

Null mutations at *dpp* lead to severe defects in gastrulation and embryonic dorsoventral polarity making examination of gut morphogenesis in these embryos difficult. Mutations in the shortvein region of *dpp*, however, allow normal gastrulation and dorsal–ventral

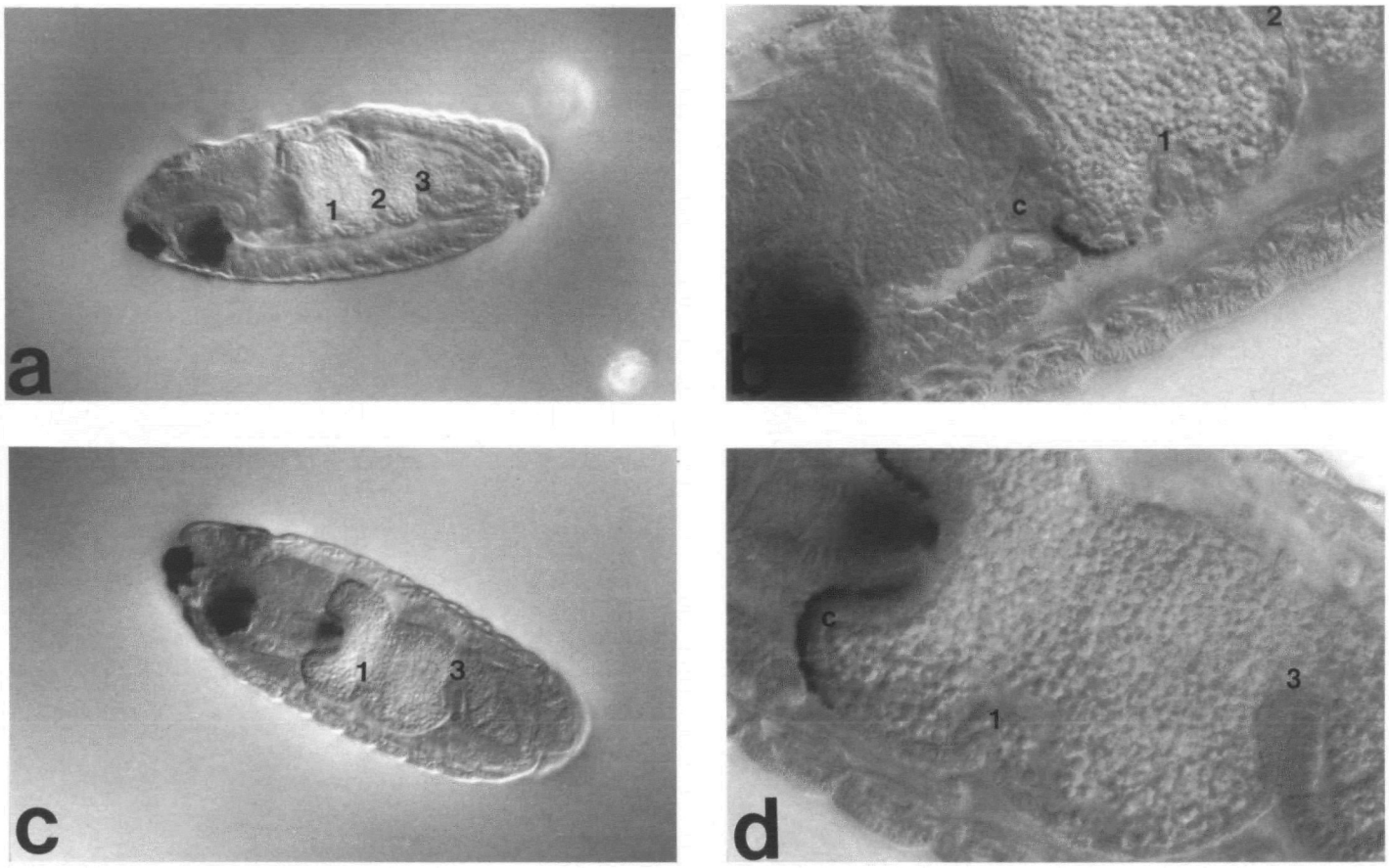

Fig. 2. Midgut morphogenesis in dpp^{S21}/dpp^{S2} embryos stained with anti-*Scr* antibody. The domain of *Scr* expression is extended anteriorly in the dpp^{shv} mutant embryo (C,D) compared to wild-type (A,B). In addition, the gastric caeca (c) arrest growth and fail to elongate and narrow, the second midgut constriction (2) does not form, and the first midgut constriction (1) is shifted posteriorly.

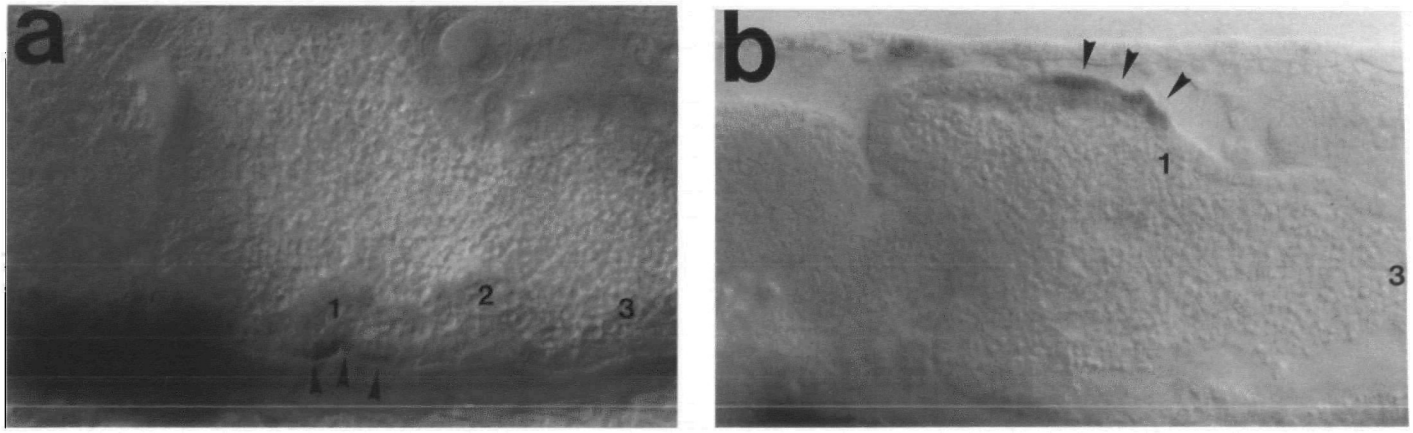

Fig. 3. *Antp* in a wild-type (A) *versus* dpp^{S21}/dpp^{S2} (B) embryo. In the wild-type embryo, *Antp* protein is found in the nuclei of the visceral mesoderm cells surrounding the first constriction (1) (A). In the dpp^{shv} mutant embryo (B), the domain of *Antp* expression and the first constriction are shifted posteriorly to approximately the position where the second constriction forms (2) in the wild-type embryo. In addition, the *Antp* domain no longer extends on either side of the first constriction but is limited to the anterior portion of the first constriction in the dpp^{shv} mutant.

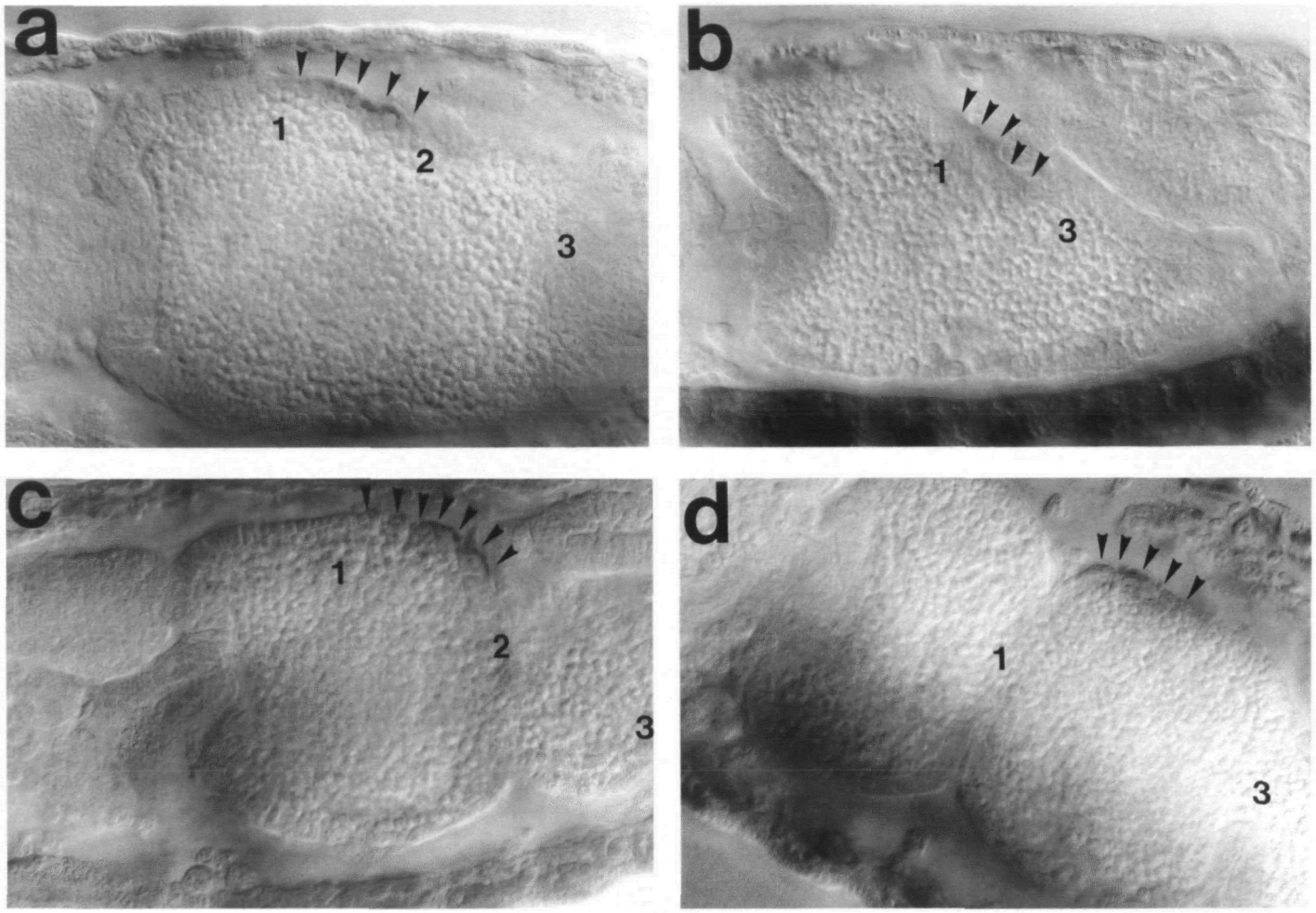

Fig. 4. *Ubx* expression in wild-type (A and C) *versus* $dpps^{21}/dpp^{S4}$ (B and D) embryos. The predominant alterations in *Ubx* expression observed in dpp^{shv} mutant embryos are a reduction in the level of *Ubx* protein in the cells which express it and/or a reduction in the number of cells expressing *Ubx*. Panels A and B show stage 15 embryos fixed and stained in the same tube. The level of *Ubx* protein is clearly reduced in the dpp^{shv} embryo (B) when compared to that of the wild-type embryo (A). Panels C and D show late stage 16 embryos also fixed and stained in the same tube. There are fewer cells in the dpp^{shv} embryo (D) expressing *Ubx* than in the wild-type control (C). Also note that the *Ubx* expression in the dpp^{shv} embryos is no longer restricted to the anterior part of the constriction which forms in PS 7 (A and C), but extends both anteriorly and posteriorly from the construction (B) or, more often, just posteriorly (D).

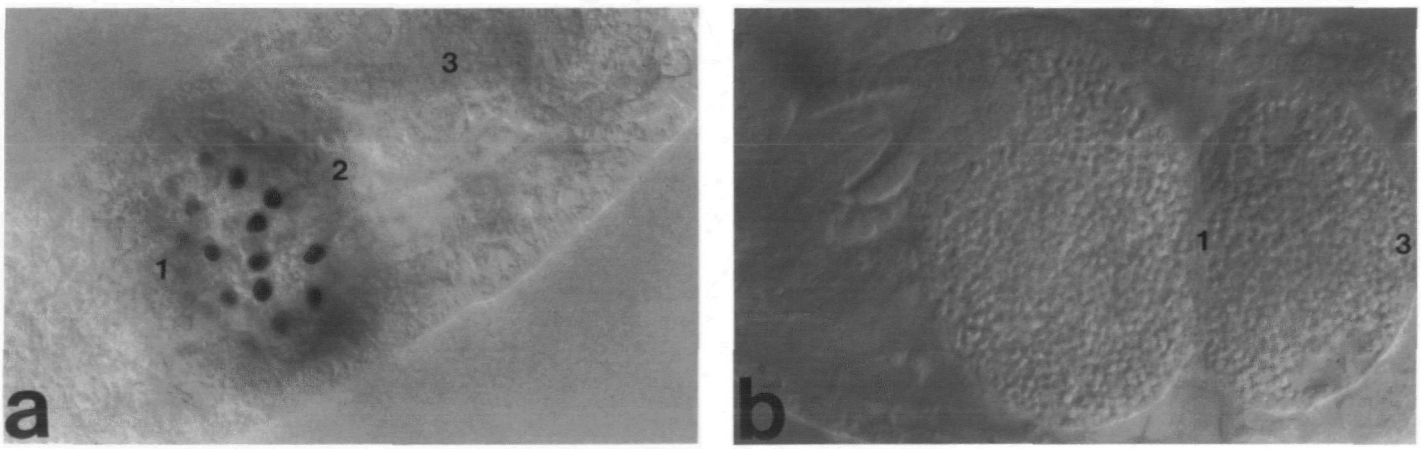

Fig. 6. *lab* protein in a wild-type embryo (A) is present in the polyploid endoderm nuclei between the first and second constrictions. No *lab* protein is detected in the endoderm nuclei of the dpp^{shv} mutant embryo (B).

pattern formation to occur but lead to larval lethality associated with slow larval growth and visible defects in internal structures, especially the Malphigian tubules (Segal and Gelbart, 1985). We have examined embryos mutant for $dpp^{shortvein}$ (dpp^{shv}) alleles for expression of *dpp* (Fig. 1B).

The *shv* region consists of more than 20 kb of DNA located 5′ to the coding region of the *dpp* gene (Fig. 1C). This region harbors four (of five known) promoters with their respective alternative 5′ non-coding exons (St. Johnston *et al.* 1990). dpp^{shv} mutant embryos were generated by crossing two different alleles to avoid making other undefined lesions on the chromosomes homozygous. A mutant embryo, dpp^{S21}/dpp^{S4}, probed with a digoxigenin labelled *dpp* DNA probe is shown in Fig. 1B. dpp^{shv} mutant embryos retain the dorsally localized blastoderm expression of *dpp* and the dorsal and lateral stripes of *dpp* in the ectoderm, but lack *dpp* expression in the midgut and have severely reduced foregut expression. Embryos mutant for dpp^{shv} alleles which eliminate only promoters P4 and P5 (dpp^{S2} and dpp^{S12}) retain some *dpp* expression along the foregut and at the caeca, however, all dpp^{shv} mutant embryos we have examined exhibit midgut defects similar to those described below. Presumably, a minimum level of *dpp* product is required for wild-type development of the visceral mesoderm. Other aspects of *dpp* embryonic function are also sensitive to the level of *dpp* product as indicated by the haploinsufficient characteristics of *dpp* (Spencer *et al.* 1982; Irish and Gelbart, 1987).

Absence of dpp *affects* Scr *expression and development of the gastric caeca*

Scr has been localized to the visceral mesoderm cells immediately posterior to the budding gastric caeca (Fig. 2A and B) (Tremml and Bienz, 1989*a*; Reuter and Scott, 1990). *dpp* is detected anterior to the *Scr* expressing cells in the visceral mesoderm of the budding caeca. Double labelling experiments have confirmed that *dpp* and *Scr* domains are non-overlapping (data not shown). Expression of both *dpp* and *Scr* in the anterior midgut is initially detected at stage 13 when the germ band is fully retracted. *Scr* expression persists through stage 17 while *dpp* expression in the caeca diminishes in stage 15 and is gone by the end of stage 16. In dpp^{shv} mutants, which have reduced *dpp* expression at the caeca, the domain of *Scr* expression is expanded anteriorly to encompass those visceral mesoderm cells that in wild-type embryos express *dpp* (Fig. 2C and D) indicating that *dpp* expression represses *Scr* expression in the visceral mesoderm cells that give rise to the gastric caeca. Caeca development is arrested in dpp^{shv} mutants: instead of extending anteriorly to form long narrow tubes, the caeca of dpp^{shv} mutants remain short and broad (Fig. 2C and D).

Absence of dpp *affects* Ubx *expression and eliminates the second midgut constriction*

In dpp^{shv} mutants, the second midgut constriction does not form but the first and third constrictions do form (Fig. 2C,D). *Antp* is expressed in the visceral mesoderm cells surrounding the first constriction of the midgut (Fig. 3A) (Tremml and Bienz, 1989*a*; Reuter and Scott, 1990), and *Ubx* is expressed in the visceral mesoderm cells of and anterior to the second constriction (Fig. 4A,4C) (Bienz and Tremml, 1988; Tremml and Bienz, 1989*a*; Reuter and Scott, 1990). dpp^{shv} mutant embryos exhibit a range of *Ubx* expression patterns. Most frequently, the domain of *Ubx* expression is narrower than in wild-type embryos (Fig. 4D) or the levels of *Ubx* protein in expressing cells are greatly reduced (Fig. 4B). We have also observed dpp^{shv} mutant embryos in which both the level of *Ubx* and the number of cells expressing it are reduced (data not shown). In addition, the position of the *Antp* domain of expression and the position of the first constriction are shifted posteriorly in dpp^{shv} mutants (Fig. 3B). These effects are identical to the effects that *Ubx* mutations have on the positions of *Antp* expression and the first constriction, indicating that the morphological defects caused by dpp^{shv} mutations are a consequence of the greatly reduced expression of *Ubx* in PS 7. However, we also find that *dpp* expression in PS 7 is reduced or absent in *Ubx* mutant embryos (Reuter *et al.* 1990), therefore the lack of either sufficient *Ubx* or *dpp* gene product could be responsible for the shift in the *Antp* expression domain and the position of the first constriction.

Extracellular dpp *protein moves from the visceral mesoderm across the endoderm*

The similarity of *dpp* to the TGF-β family of growth factors (Padgett *et al.* 1986) and the fact that it is secreted from *Drosophila* cells in culture (Panganiban *et al.* 1990) led us to examine whether *dpp* might move from its sites of synthesis in the embryo. In the PS 7 region of the developing midgut, *dpp* RNA is detected in the visceral mesoderm cells at and anterior to the developing second constriction at stages 11–17 (Fig. 5A,5B, and data not shown) (stages according to Campos-Ortega and Hartenstein, 1985). Polyclonal antibodies to *dpp* (Panganiban *et al.* 1990) detect protein in and around these same visceral mesoderm cells at stage 14 (Fig. 5C). By stage 16, *dpp* protein is also detected surrounding the underlying endoderm cells (data not shown). By stage 17, most of the *dpp* protein is concentrated at the luminal surface of the endoderm cells (Fig. 5D), while the RNA can still be detected only in the mesoderm (Fig. 5B). This indicates that *dpp* protein, secreted from visceral mesoderm cells, moves from its site of synthesis across at least one cell layer. The fact that the site of synthesis is the visceral mesoderm and the adjacent cells are endoderm, raised the intriguing possibility that *dpp* might be involved in intercellular communication or inductive interactions between the two germ layers.

Absence of dpp *in the visceral mesoderm eliminates* lab *expression in the endoderm*

The homeotic gene *lab* is expressed in the endodermal cells between the first and second constrictions

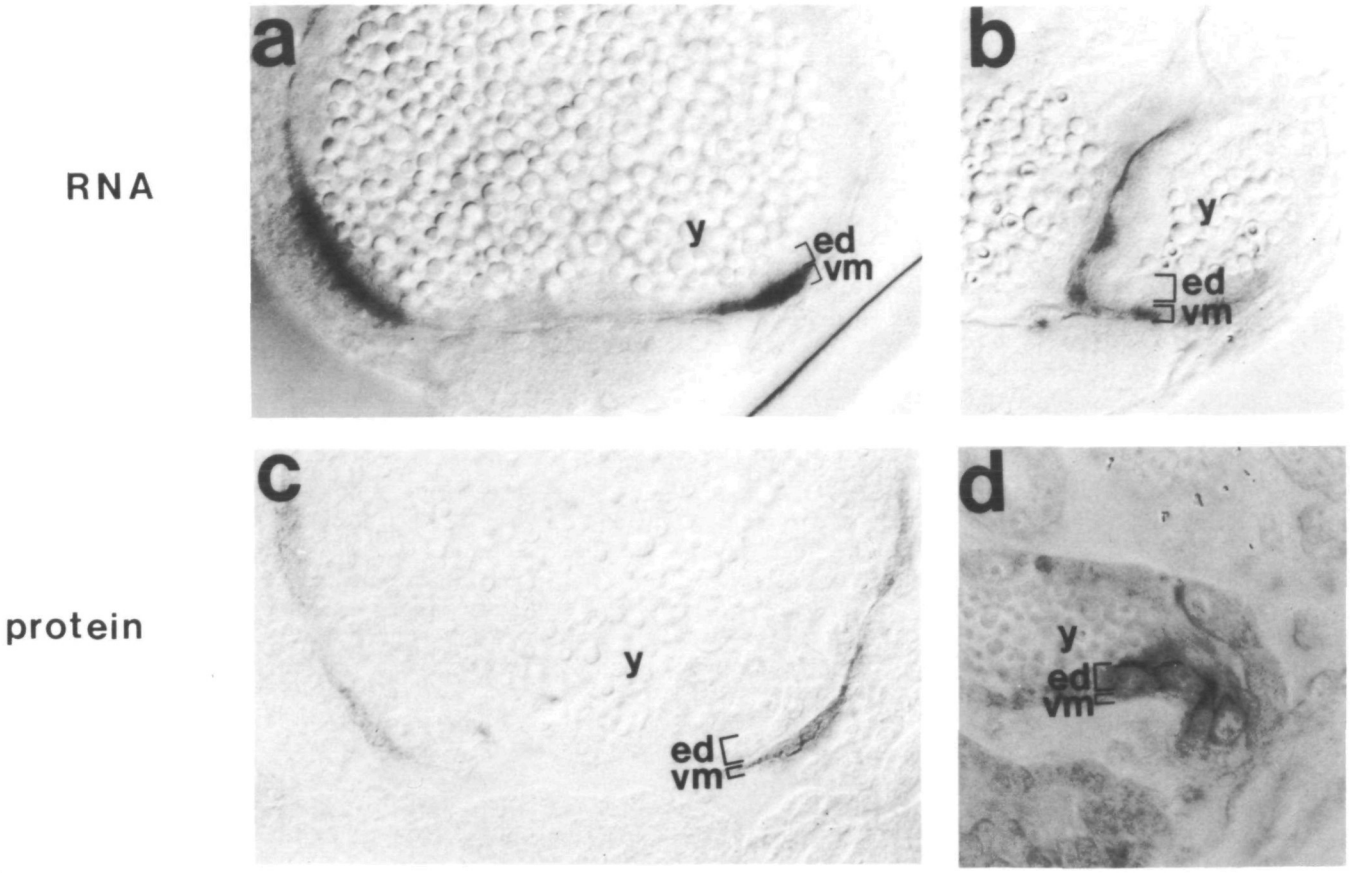

Fig. 5. *dpp* RNA (A and B) *versus* protein (C and D) localization at and anterior to the second midgut constriction in 6 micron sagittal sections of stage 14 (A and C) and 17 (B and D) wild-type embryos photographed at 100×. While *dpp* RNA remains localized to the visceral mesoderm cells (vm), *dpp* protein migrates through the endoderm (ed) cells and by stage 17 is concentrated at the apical surface of the endoderm cells adjacent to the yolk (y).

(Diederich *et al.* 1989; and Fig. 6A), directly beneath the visceral mesoderm cells that express *Ubx* and *dpp*. Minor defects in second constriction formation have been reported to occur in *lab* mutants (Immergluck *et al.* 1990). In dpp^{shv} mutant embryos, *lab* expression is absent from the endodermal cells of the midgut (Fig. 6B) indicating that the migration of the *dpp* protein from the mesoderm across the endoderm (Fig. 5) may induce the expression of *lab* in the underlying endoderm.

Mutations in abdA *expand the domains of expression of* Ubx, dpp, *and* lab

The *abdA* gene is expressed in the midgut visceral mesoderm in parasegments 8–12 (Tremml and Bienz, 1989*a*; Karch *et al.* 1990) and inhibits the expression of *Ubx* in the posterior midgut (Bienz and Tremml, 1988). Although wild-type expression of *Ubx* (Fig. 7A) and *dpp* (Fig. 7C) are limited to the PS 7 visceral mesoderm, their expression in an *abdA* mutant embryo is no longer restricted to PS 7, but extends over the entire posterior midgut (Fig. 7B,D). To determine whether the ectopic expression of *dpp* in the visceral mesoderm induces ectopic expression of *lab* in the adjacent endodermal cells, we examined the *lab* expression in *abdA* mutant embryos. In contrast to the wild-type pattern of *lab* expression (Fig. 7E), we find that the domain of *lab* expression is extended in the *abdA* mutants to include the endoderm of the posterior midgut directly beneath the visceral mesoderm cells that ectopically express *dpp* (Fig. 7F).

Discussion

Similarities in the expression patterns of *dpp* and *Ubx* in the visceral mesoderm led us to examine the regulatory relationships between *dpp* and homeotic genes during midgut development. We have identified three responses to *dpp* in the development of the *Drosophila* midgut that involve homeotic gene expression. The significance of these regulatory relationships to morphogenesis is best indicated by the fact that mutations in *dpp* alter the same structures, the gastric caeca and the second constriction, affected by mutations in two of the homeotic genes regulated by *dpp*. That *dpp* functions as a secreted factor is best illustrated by its regulation of a third homeotic gene across germlayers.

dpp *regulation of* Scr *expression and gastric caeca formation*

dpp negatively regulates *Scr* expression in the visceral

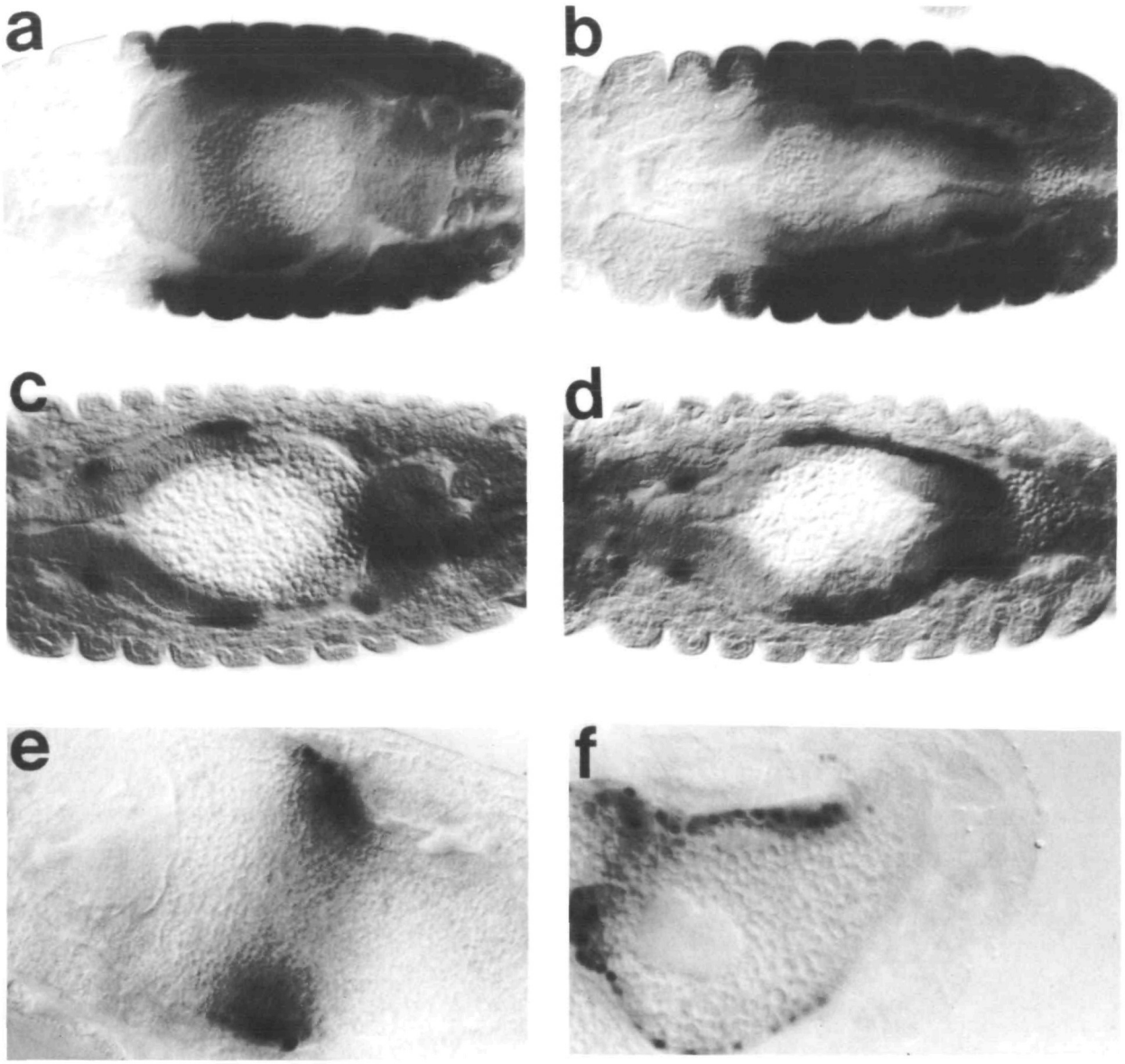

Fig. 7. *Ubx*, *dpp* and *lab* expression in *abdA* mutant embryos. *Ubx* (A), *dpp* (C), and *lab* (E) are normally expressed in the central region of the midgut. *abdA* is normally expressed in the visceral mesoderm cells of the posterior midgut (Karch *et al.*). In $abdA^{M1}$ mutant embryos, the domains of expression of both *Ubx* (B) and *dpp* (D) are expanded to include the visceral mesoderm cells in the posterior midgut (pm) as well. When *dpp* is ectopically expressed in the visceral mesoderm of the posterior region of the midgut due to a mutation in *abdA*, the expression of *lab* is activated in the endoderm cells of the posterior region of the midgut (F). Panels A–D are dorsal views photographed at 40×; E and F are lateral views photographed at 63×.

mesoderm cells that become the caeca (Fig. 8). In dpp^{shv} mutants, the domain of *Scr* expression at the gastric caeca is extended anteriorly to include those cells that normally express *dpp*. The ectopic expression of *Scr* in these cells may prevent the cells from evaginating as caeca. Alternatively, ectopic *Scr* expression by itself may not be sufficient to block development of the caeca but may serve as an indicator that the cell fate at the anterior end of the midgut is changed in the absence of *dpp*. The cell fate indicated by *Scr* expression must then be incompatible with the processes needed to form caeca. The mechanism by which *dpp* normally represses *Scr* expression in the anterior midgut and permits development of the caeca remains to be determined.

Although ectopic expression of *Scr* blocks the development of caeca, normal expression of *Scr* immediately posterior to the cells forming the caeca is required for caeca development (Reuter and Scott, 1990). As *dpp* expression at the anterior end of the midgut is not affected in the *Scr* mutant embryos, *dpp* expression, while required, is not sufficient for caeca development. The two adjacent regions of visceral mesoderm cells at the anterior end of the midgut, one

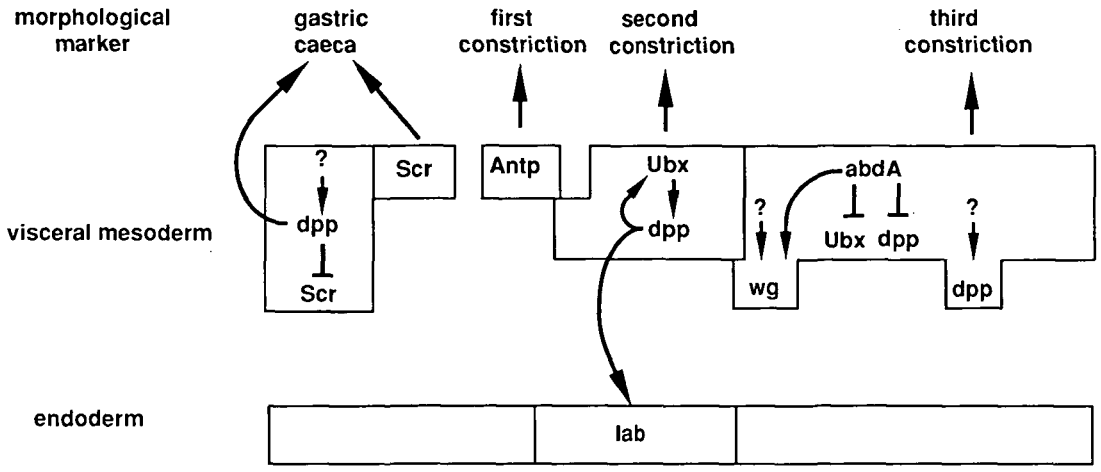

Fig. 8. The interactions between *dpp* and homeotic genes during midgut morphogenesis. Arrows indicate positive regulation and inverse tees indicate negative regulation. *dpp* expression in the gastric caeca precludes *Scr* expression there, since the *Scr* domain is extended anteriorly in embryos lacking *dpp* expression in the caeca. *dpp* expression is unaffected by a lack of *Scr*, however, both *dpp* and *Scr* are required for appropriate development of the caeca. In the absence of either *dpp* or *Scr*, the caeca do not form properly, but the defects are distinct for each mutant (See text and Reuter and Scott, 1990 for details). *Ubx* and *dpp* are expressed in the visceral mesoderm cells between the first and second midgut constrictions. Both are required for the formation of the second constriction and stimulate expression of one another. Between the first and second constrictions, *dpp* migrates from the visceral mesoderm cells which synthesize it through the underlying endoderm cells, stimulating expression of the *lab* gene in those cells. The *abdA* gene is expressed by visceral mesoderm cells of the posterior region of the midgut. *abdA* represses the expression of *Ubx* and *dpp* in the cells where it is expressed, as in the absence of *abdA* function the *Ubx* and *dpp* domains are extended posteriorly. *wg* expression is activated in the visceral mesoderm posterior to the second constriction by *abdA*. Regulation of *wg* expression requires other factors as *wg* is not expressed throughout the *abdA* domain. *dpp* expression is activated in a third domain in the mesoderm of the midgut, at the third constricition, by a mechanism that does not require *Ubx* and that is not down-regulated by *abdA*.

expressing *dpp* and the other expressing *Scr*, must cooperate to direct the formation of the gastric caeca.

dpp *and* Ubx *regulate one another*

Wild-type expression of both *dpp* and *Ubx* is crucial for development of the midgut (Fig. 8) because in the absence of either *Ubx* or *dpp* the second constriction does not form. These two genes positively regulate the expression of one another: in the absence of *dpp* function in the gut, *Ubx* expression is reduced, but not absent, and in the absence of *Ubx* function, *dpp* expression is reduced or absent depending on the *Ubx* allele (See Reuter *et al.* 1990 for discussion of the regulation of *dpp* expression by *Ubx*). This is in contrast to what has been reported by Immergluck *et al.* (1990) who report a unidirectional effect of *Ubx* on *dpp*. By analogy to the relationship between *dpp* and *zen* (Rushlow *et al.* 1987; Rushlow and Levine, 1990), we predict that *Ubx* is required for the initiation of *dpp* expression in PS 7, and that *dpp* is in turn required to maintain expression of *Ubx*. This prediction is consistent with the range of *Ubx* expression phenotypes we observe in dpp^{shv} mutant embryos.

The requirement for the coordinated activities of *Ubx* and *dpp* is illustrated by the posterior extension of *Antp* expression and the posterior shift of the first constriction in embryos mutant for either *dpp* or *Ubx*. As expression of both *dpp* and *Ubx* is regulated negatively by *abdA* (Fig. 7) it is of interest to determine whether *abdA* acts on both genes independently or whether, for example, the effects of *abdA* on *Ubx* expression are mediated by *abdA*'s negative regulation of *dpp* expression. When both *abdA* and *Ubx* are expressed in the same cells, *abdA* can still repress *dpp* expression (Reuter *et al.* 1990) but it is not yet known whether *abdA* can also repress *Ubx* in the presence of ectopic *dpp* expression.

dpp *migration across germ layers affects* lab *expression*

One of the most interesting results of these experiments is that the *dpp* protein, after migrating from the visceral mesoderm across the endoderm, may induce the expression of the *lab* gene in the endoderm cells. In support of this mechanism, ectopic expression of *dpp* in the visceral mesoderm cells in *abdA* mutant embryos gives rise to a parallel ectopic expression of *lab* in the endoderm of the posterior midgut. Because *Ubx* and *dpp* are colocalized in the part of the visceral mesoderm of wild-type embryos that overlies the *lab*-expressing endoderm, and because *Ubx* is also ectopically expressed in the posterior midgut of *abdA* mutants, it remains a formal possibility that *Ubx* acts *via* a *dpp*-independent pathway to stimulate *lab* expression in the adjacent tissue. We believe this is unlikely because in dpp^{shv} mutants, which retain some *Ubx* expression, *lab* is not expressed in the underlying endoderm.

The fact that *dpp* is a secreted protein makes it a

strong candidate for regulation of gene expression between germlayers. However, the movement of *dpp* is quite limited. We do not observe extracellular *dpp* protein moving into regions of the visceral mesoderm that do not make *dpp* mRNA and cannot detect it migrating into or across the endoderm at the anterior end of the midgut. Perhaps *dpp* is associated with other extracellular proteins which limit its movement. We have observed that *dpp* protein produced in a *Drosophila* cell culture expression system associates with other proteins on the surface of the tissue culture dish (Panganiban *et al.* 1990). The *Drosophila wingless* gene product also appears to exhibit a limited potential for movement from the cells in which it is made (van den Heuvel *et al.* 1989). However, even the limited movement of extracellular *dpp* may allow it to exert an influence in a specific domain, for example, PS 7 of the visceral mesoderm, such that the intercellular communication mediated by *dpp* stabilizes a specific cell fate decision in that domain. This mechanism allows a population of cells to cooperate in maintaining the cell fate of the population.

dpp *exerts pleiotropic effects on homeotic gene expression and midgut morphogenesis*

The developmental origin or the environment of cells must determine how cells respond to *dpp* because, as reported here, at least three different homeotic genes can respond to the presence of *dpp* in the midgut. The only previous report of a change in gene expression due to *dpp* dealt with the failure to maintain *zen* expression in the amnioserosa of *dpp* null animals (Rushlow and Levine 1990); interestingly, *zen* also encodes a homeodomain containing protein (Rushlow *et al.* 1987). The differences in homeotic gene expression between the *dpp*-expressing regions of the midgut are paralleled by morphogenetic differences in these regions of the gut. In one *dpp* domain, the caeca are formed, while in the other a constriction is formed. Although *dpp* and *Ubx* are necessary to form the second constriction, they are not sufficient for constriction formation, as is illustrated by the absence of the second and third constrictions in *abdA* mutant embryos in which *Ubx* and *dpp* are expressed throughout the posterior midgut. Therefore, the effects of *dpp* on both gene expression and morphogenetic events are specific in different positions in the embryo. We propose that expression of *dpp* is necessary, but not sufficient, for specific aspects of homeotic gene expression and morphogenesis required to form the *Drosophila* midgut. The mechanisms by which the domains of *dpp* and homeotic gene expression are initially established in the visceral mesoderm remain to be determined. Clearly, once expressed, the genes regulate one another.

We thank Tom Kaufman for providing antibody to *labial* and Sean Carroll for critically reading the manuscript. Support for this research was provided by grants from the American Cancer Society to F.M.H., Cancer Center Core Support from NCI to H.C. Pitot, NIH Training Grant GM07215 for G.E.F.P., NIH Grant 18163 to M.P.S. M.P.S. is an Investigator of the Howard Hughes Medical Institute. This paper is dedicated to the memory of Calvin Calmon.

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(*Accepted 9 October 1990*)