Development 120, 2799-2809 (1994)
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Role of the *teashirt* gene in *Drosophila* midgut morphogenesis: secreted proteins mediate the action of homeotic genes

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SUMMARY

Homeotic genes control the development of embryonic structure by coordinating the activities of downstream 'target' genes. The identities and functions of target genes must be understood in order to learn how homeotic genes control morphogenesis. *Drosophila* midgut development is regulated by homeotic genes expressed in the visceral mesoderm, where two of their target genes have been identified. Both encode secreted proteins. The *Ultrabithorax* (*Ubx*) homeotic gene activates transcription of the *decapentaplegic* (*dpp*) gene, which encodes a TGFβ class protein, while in adjacent mesoderm cells the *abdominal-A* (*abd-A*) homeotic gene activates transcription of the *wingless* (*wg*) gene, which encodes a Wnt class protein. The homeotic genes *Antennapedia* (*Antp*) and *Sex combs reduced* (*Scr*) act in more anterior midgut regions. Here we report the identification of another homeotic gene target in the midgut mesoderm, the *teashirt (tsh)* gene, which encodes a protein with zinc finger motifs. *tsh* is necessary for proper formation of anterior and central midgut structures. *Antp* activates *tsh* in anterior midgut mesoderm. In the central midgut mesoderm *Ubx*, *abd-A*, *dpp*, and *wg* are required for proper *tsh* expression. The control of *tsh* by *Ubx* and *abd-A*, and probably also by *Antp*, is mediated by secreted signaling molecules. By responding to signals as well as localized transcription regulators, the *tsh* transcription factor is produced in a spatial pattern distinct from any of the homeotic genes.

Key words: homeotic genes, morphogenesis, midgut, *decapentaplegic*, *wingless*, TGFβ

INTRODUCTION

Conversions of one part of the body into a copy of another result from mutations in homeotic genes. The dramatic phenotypes resulting from loss of homeotic gene function or from ectopic production of homeotic proteins demonstrate the powerful organizing functions of the proteins (Duncan, 1987; Kaufman et al., 1990). Many of the *Drosophila* homeotic genes are present in two clusters, the Antennapedia complex (ANT-C) and the Bithorax complex (BX-C). The two clusters are thought to have arisen from a split in a single ancestral complex, and are collectively termed the homeotic complex, HOM-C. HOM-C genes all encode transcription regulators containing a DNA-binding homeodomain. The fly homeotic genes are expressed in restricted domains along the anterior-posterior axis where they are able to organize cells to form segment-specific structures (Duncan, 1987; Mahaffey and Kaufman, 1988). Related HOM-C gene clusters have been identified in organisms as diverse as humans and the nematode *C. elegans* and are presumably present in all animals (McGinnis and Krumlauf, 1992). In mice and humans there are four clusters of homeotic genes, called Hox genes, each of which appears to be related in spatial expression, protein sequence, and function with the HOM-C fly genes. To some extent the genes are functionally interchangeable between species; three Hox genes act like their fly homologs in ectopic expression experiments in flies (Malicki et al., 1990; McGinnis et al., 1990; Zhao et al., 1993). Homeotic genes are responsible for regional specification of cell fates in all organisms where their functions have been studied.

Homeotic proteins regulate sets of target genes, thus causing cells to follow developmental paths appropriate to their position in the animal. How are target genes regulated by the homeotic transcription factors? Do the proteins regulate similar sets of target genes differently, or are the targets of each protein different? To what extent are the effects of homeotic proteins tissue-specific or stage-specific? Are the effects of homeotic proteins upon their targets cell autonomous or are cells influenced at a distance? The answers to these questions are central to understanding the roles homeotic proteins play in pattern formation. Only a few target genes have been identified to date (Andrew and Scott, 1992; Botas, 1993) and more must be found to learn how they work together in development.

The relative simplicity of *Drosophila* midgut morphology

makes it a useful tissue for examining the effects of homeotic genes, and for identifying downstream target genes. The midgut is formed late in embryogenesis and is a tube consisting of two cell layers, an inner endodermal layer and an outer mesodermal layer, encasing the yolk (Skaer, 1993). The gut becomes divided into several compartments through the constriction of the midgut in three places, and four tubes called gastric caeca evaginate from the anterior of the midgut. Four homeotic genes, each one expressed in a distinct and non-overlapping region of the visceral mesoderm, control constriction and caeca formation (Bienz and Tremml, 1988; Tremml and Bienz, 1989; Reuter and Scott, 1990). The homeotic genes regulate both cell shape changes and movements necessary for morphogenesis; no cell divisions occur during these late patterning events.

Two targets of homeotic gene regulation in the visceral mesoderm, *decapentaplegic* (*dpp*) and *wingless* (*wg*) appear to be used for communication between the two midgut cell layers. Both *dpp* and *wg* are transcribed in the mesoderm, but their protein products are secreted signaling molecules that move into the endoderm (van den Heuvel et al., 1989; Panganiban et al., 1990b; Reuter et al., 1990). Both *dpp* and *wg* are required for normal expression of the homeotic gene *labial* (*lab*) in the endoderm (Immerglück et al., 1990; Reuter et al., 1990; Tremml and Bienz, 1992). Thus, the endoderm is locally induced to differentiate in response to homeotic genes acting in the overlying mesoderm. The *dpp* and *wg* genes are necessary for morphogenesis; mutations in either gene prevent formation of the central midgut constriction. A fourth gene that responds to homeotic genes, *pdm-1*, encodes a protein belonging to the POU class (Affolter et al., 1993). *pdm-1* transcripts are initially expressed throughout the endoderm, then the transcripts disappear from two regions of the endoderm. *Ubx* represses *pdm-1* in the central endoderm, but unlike the induction of *lab* in this same region, the repression of *pdm-1* does not require *dpp*. Thus additional signals from mesoderm to endoderm are regulated by *Ubx*.

We have identified a fifth gene that responds to homeotic genes in the midgut, the *teashirt* (*tsh*) gene. Previous studies have shown that *tsh* is expressed in the epidermis of the thorax and abdomen, where it promotes the development of trunk structures and represses the formation of head structures (Fasano et al., 1991). The expression of *tsh* is modulated in the epidermis by *Antp* and the BX-C genes (Röder et al., 1992). Here we demonstrate that *tsh* is required for the formation of two of the three midgut constrictions and is regulated by three homeotic genes in the visceral mesoderm. The regulation of *tsh* by the homeotic genes in the midgut is mediated by *dpp* and *wg*, indicating roles for both transcription factors and secreted proteins in *tsh* regulation.

MATERIALS AND METHODS

Fly stocks

Stocks are described by Lindsley and Zimm (1992) and additional references as follows: Scr^{W17} (Wakimoto and Kaufman, 1981) makes no detectable *Scr* protein in epidermis or mesoderm, but some *Scr* protein is detectable in the nerve cord (Riley et al., 1987; M. Petitt, unpublished observation). $Antp^{W10}$ (Wakimoto and Kaufman, 1981) produces no detectable *Antp* protein (Carroll et al., 1986). $Ubx^{6.28}$ homozygous mutants make no detectable *Ubx* protein due to a 32 bp deletion in the 5′ exon that results in premature translation stop (Beachy et al., 1985; Weinzeirl et al., 1987). $abd\text{-}A^{MX1}$ (Sanchez-Herrero et al., 1985) makes no detectable *abd-A* protein (Karch et al., 1990). $abd\text{-}A^{D24}$ is associated with an apparent point mutation with decreased *abd-A* protein staining (Karch et al., 1990) and fails to form the two posterior midgut constrictions (L.D.M., unpublished observation). dpp^{s4}, dpp^{s6} (Segal and Gelbart, 1985) are mutations in the shortvein region, which allow normal gastrulation but have no detectable *dpp* mRNA in PS7 of the midgut (Panganiban et al., 1990b). wg^{IL114} (Nüsslein-Volhard et al., 1984) is a temperature-sensitive allele. At 25°C wg^{IL114} has the same effect as a *wg* null mutation (Baker, 1988). tsh^{8} (Fasano et al., 1991) is a deficiency that removes at least 40 kb of DNA and creates a null allele of the gene. *TW161* (Wright et al., 1976) is a large deficiency that removes the DNA from 38A6-B1 to 40A4-B. HS-*dpp* (R.W. Padgett, R.K. Blackman, and W. M. Gelbart, unpublished data) is a fusion of the *dpp* cDNA to a *Drosophila* heat shock-inducible promoter. HS-*wg* is a similar construct encoding the complete wild-type *wg* protein (Noordermeer et al., 1992). *Antp-lacZ* contains 16 kb of the *Antp* P1 promoter fused to *lacZ* (M. Petitt and M. P. S., unpublished data). Enhancer trap line l(2)2657 (Karpen and Spradling, 1992) is an insert in the *wg* gene that is expressed in PS8 of the visceral mesoderm (L. D. M., unpublished observation).

Sources and preparation of antibodies

The rat polyclonal antibody to *tsh* has been described previously (Zeng et al., 1993) and was used at a dilution of 1:500. A polyclonal rabbit antibody to muscle myosin was the gift of D. Kiehart and was used at a dilution of 1:500. A polyclonal rabbit antibody to *wg* was the gift of R. Nusse and was used at a dilution of 1:100 (van den Heuvel et al., 1989). Partially purified polyclonal antibody to *dpp* protein was a gift from F. M. Hoffmann and was used at 1 μg/ml (Panganiban et al., 1990a). The *Scr* monoclonal cell line 6H4 (Glicksman and Brower, 1988) was a gift from D. Brower. Ascites fluid was prepared from the 6H4 cell line and used at a dilution of 1:1000.

Embryo fixation and immunohistochemical staining

Embryos were dechorionated, fixed and devitellinized essentially as described in Reuter and Scott (1990). Dechorionated embryos were washed in 0.1% Triton X-100 and transferred to Eppendorf tubes containing 200 μl HME with 4% formaldehyde (HME : 50 mM Hepes pH 6.9, 1 mM EGTA, 2 mM $MgSO_4$) and 800 μl heptane. Embryos were fixed for 25-35 minutes at 22°C by rotating end-over-end. Embryos to be stained with antibody to *dpp* were fixed for 35 minutes to improve the staining. The fixative phase was removed and the embryos devitellinized by shaking vigorously with 700 μl of methanol for 1-2 minutes. The devitellinized embryos were rinsed in methanol, then methanol with 0.3% H_2O_2 for 2 minutes, then rinsed in methanol to remove the H_2O_2. After one rinse in 1:1 methanol: PBSTB, the embryos were blocked for at least 3 hours in PBSTB with changes approximately every 30 minutes (PBSTB : 1× PBS, 0.2% BSA, 0.1% Triton X-100). The embryos were incubated with an appropriate dilution of primary antibody (noted above) overnight at 4°C on a tube rocker. After washing for at least three hours with several changes of PBSTB (one wash in PBSTB with 5% normal goat serum), the embryos were incubated overnight at 4°C, on a tube rocker, with a secondary antibody conjugated to biotin (Jackson Labs, Vector Labs). All biotin-conjugated secondary antibodies were pre-adsorbed for several hours against wild-type embryos at a dilution of 1:50 and used at a final dilution of 1:500. After washing for at least 3 hours with several changes of PBSTB, the embryos were incubated with a biotin-avidin-HRP complex (Vector labs; Vectastain Elite) for 1 hour and washed again for at least 3 hours in PBSTB. Staining was performed in DAB solution (DAB solution : 0.5 mg/ml diaminobenzidine, 100 mM Tris, pH 7.5, 0.003% H_2O_2) and stopped with several washes in PBSTB. Embryos for double-label experiments were treated as above except that 0.4% $NiCl_2$ was included in the DAB solution to produce

a blue-black precipitate in the first stain. The embryos were then washed several times in PBSTB and treated with 0.3% H_2O_2 in PBSTB to destroy any remaining peroxidase activity. The second staining was performed as described above (without $NiCl_2$ in the DAB solution) to yield a brown precipitate. Stained embryos were dehydrated through 30%, 50%, 70% and 2× 95% ethanol, then mounted in methyl salicylate.

Immunohistochemical staining and in situ hybridization

Embryos were dechorionated, fixed, and devitellinized as described above except that PBTH was used instead of PBT (PBTH : 1× PBS, 0.1% Tween 20, 50 μg/ml heparin, 100 μg/ml tRNA). Nuclease-free BSA at 1% was included in one of the washes between each antibody incubation to block. Antibody incubations were performed in the presence of 0.1 U/μl RNasin (Promega). After reaction with DAB, the embryos were rinsed several times with PBT (PBT : 1× PBS, 0.1% Tween 20). In situ hybridizations were performed essentially as described in Tautz and Pfeiffle (1989). The embryos were fixed for 20 minutes in 4% formaldehyde in PBT, then rinsed several times with PBT. The embryos were digested with 50 μg/ml proteinase K for 2 minutes and stopped with 2 mg/ml glycine for 2 minutes. The embryos were post-fixed for 20 minutes in 4% formaldeyde in PBT, followed by several rinses in PBT. After rinses in 1:1 PBT:hybridization buffer and straight hybridization buffer, the embryos were pre-hybridized overnight at 60°C (hybridization buffer : 50% deionized formamide, 5× SSC, 100 μg/ml sonicated herring DNA, 50 μg/ml heparin, 0.1% Tween 20). Digoxigenin-labeled anti-sense RNA for the *dpp* cDNA (Padgett et al., 1987) was generated using T3 RNA polymerase and the Bluescript vectors (Stratagene). In situ hybridizations were performed overnight at 60°C. The embryos were washed at 60°C with hybridization buffer and 1:1 hybridization buffer:PBT for 20 minutes each, followed by 3 washes for 20 minutes with PBT at 22°C. Anti-digoxigenin antibody conjugated to HRP was pre-adsorbed against wild-type embryos, then used at a dilution of 1:2000 in PBT for several hours at 22°C. After washing for at least 2 hours in PBT with changes every 20 minutes, the embryos were developed as above with 0.4% $NiCl_2$. Stained embryos were dehydrated through 30%, 50%, 70% and 2× 95% ethanol, then mounted in methyl salicylate.

Heat shock protocols

HS-*wg* and HS-*dpp* : 12-hour embryo collections were placed at 37°C for 30 minutes three times with 30 minutes at 22°C between each heat shock, allowed to recover for 3 hours at 22°C, then fixed as described for immunohistochemical staining.

HS-*dpp*; HS-*wg* : embryos were collected for 12 hours from a cross of HS-*dpp* virgins to HS-*wg* males and then treated as above.

wg^{IL114} : 12-hour collections were taken at 18°C then the embryos were transferred to 25°C for 6 hour and fixed as above.

HS-*dpp*; wg^{IL114} : 12-hour collections were taken at 18°C and then the embryos were transferred to 25°C for 1 hour prior to heat shocking three times for 30 minutes with 30 minutes at 25°C between each heat shock. After a 3-hour recovery period at 25°C they were fixed as above.

Microscopy and photography

The stained embryos were mounted in methyl salicylate and examined using DIC optics on a Zeiss Axiophot. Photographs were taken on Ektachrome 64 film using a blue filter. Color slides were scanned with a Nikon scanner and prepared as figures using Adobe Illustrator 5.0.

RESULTS

teashirt function is required for proper midgut morphogenesis

The anterior and posterior primordia of the midgut endoderm form by the invagination of cells at the poles of the blastoderm embryo (Campos-Ortega and Hartenstein, 1985; Tepass and Hartenstein, 1994). The cells migrate toward each other along the germband (stages 7-12) and eventually fuse bilaterally during stage 13 (stages according to Campos-Ortega and Hartenstein, 1985). Also during stage 13, a layer of mesoderm attaches to the endoderm. The two midgut cell layers then close ventrally and dorsally to encircle the yolk by stage 14. During stages 15-16, the midgut tube is divided into four compartments by the formation of three constrictions along the antero-posterior axis. Also during stages 14-16, four tubes called the gastric caeca evaginate from the anterior of the midgut. The structure of the embryonic midgut is most easily examined in stage 16 embryos using an antibody to muscle myosin. Muscle myosin is produced in all visceral mesoderm cells prior to formation of the constrictions (Fig. 1A), providing a clear view of midgut morphology.

The *tsh* gene is required for correct morphogenesis of the midgut, consistent with its expression in the visceral mesoderm (shown below and in Fasano et al., 1991). Two deficiencies, tsh^8 and *TW161*, that cover the *tsh* gene were used to examine *tsh* function in the midgut (Wright et al., 1976; Fasano et al., 1991). Both tsh^8/tsh^8 and tsh^8/*TW161* embryos fail to form the central constriction and often contain a misplaced anterior constriction (Fig. 1B). In 87% of the mutant animals, an indentation forms close to the gastric caeca; occasionally a small anterior compartment is evident (data not shown). The remaining 13% of *tsh* mutants form an apparently normal anterior constriction. The central constriction is normally the first of the three to form, so mutant *tsh* homozygous embryos can easily be recognized because they usually form only the posterior constriction. In stage 16 embryos, an indentation at the position of the normal central constriction is sometimes present, although the constriction never fully forms. Thus *tsh* plays an essential role in the morphogenesis of structures whose formation is also regulated by the homeotic genes.

teashirt protein distribution in the embryonic midgut, and its relationship to other regulators

The expression of *tsh* protein in the developing midgut was examined using a rat polyclonal antiserum. This serum is specific for *tsh* protein; it does not detect any signal in embryos homozygous for a *tsh* null mutation (data not shown). *tsh* protein is first detected in the visceral mesoderm late in stage 12. The homeotic and *tsh* proteins are present prior to formation of the gut constrictions (Fig. 2 and Tremml and Bienz, 1989; Reuter and Scott, 1990). *tsh* protein accumulates to high levels in the mesoderm in two domains, one in the anterior midgut roughly where *Antp* is expressed and one in the central part of the midgut near the boundary between *Ubx* and *abd-A* expression (Fig. 2A). *tsh* protein is also detectable at very low levels in more posterior visceral mesoderm (Fig. 2A,B). A higher magnification of a dissected midgut at Stage 14 demonstrates the extent of the two main visceral mesoderm domains (Fig. 2B). The anterior domain of *tsh* expression is about 14 nuclei long along the anterior-posterior axis, while the posterior domain is approximately six nuclei long. Later, beginning at stage 15, *tsh* expression is observed in the endodermal cells that will make up the second midgut compartment, shown here at stage 16 (Fig. 2C). The two *tsh* domains overlap with homeotic gene expression

domains, but have different boundaries, as is summarized in Fig. 2D (demonstrated below).

To define more precisely the mesoderm expression domains, wild-type embryos were double-labeled for *tsh* protein and homeotic proteins. *Scr* is expressed in a domain of about four nuclei along the anteroposterior axis in cells near the developing gastric caeca. *Antp* is expressed in a ten cell domain posterior to *Scr*. The *Scr* and *Antp* domains are always separated by approximately two cells, which contain no detectable *Scr* or *Antp* protein (Reuter and Scott, 1990). The overlap of *Antp* protein and *tsh* protein is most easily demonstrated using an *Antp* P1 promoter fragment fused to *lacZ*, which is expressed in the same position as *Antp* itself (M. Petitt and M. P. S., unpublished data). The β-galactosidase is located in the cytoplasm, making it distinguishable from the nuclear *tsh* signal. The posterior border of *tsh* expression is coincident with the posterior border of *Antp-lacZ* expression (Fig. 3B). At the anterior edge of the *tsh* patch, about four nuclei have *tsh* protein but no detectable β-galactosidase protein. Thus *tsh* expression protrudes anterior to the *Antp* domain. In a double-label experiment, all cells express either *tsh* or *Scr* protein (Fig. 3A), so *tsh* protein overlaps with *Antp* and spans the gap between *Scr* and *Antp* in the anterior visceral mesoderm.

The posterior patch of *tsh* expression is near the junction between *Ubx* and *abd-A*-expressing cells, where the second midgut constriction forms. There is a sharp boundary between the *Ubx*- and *abd-A*-expressing cells. No cells produce both *Ubx* and *abd-A* proteins, in part because *abd-A* is a strong negative regulator of *Ubx* transcription (Tremml and Bienz, 1989). The homeotic proteins and *tsh* protein are both nuclear, so the expression of *tsh* in this region was located relative to *dpp* protein. *tsh* and *dpp* are detected in approximately complementary domains in the visceral mesoderm (Fig. 3C). *dpp* is a secreted protein, so to compare sites of transcription it is necessary to compare the distributions of *tsh* protein and *dpp* RNA. The locations of *tsh* protein and *dpp* RNA are largely non-overlapping, but one to two cells contain *tsh* protein and *dpp* RNA (Fig. 3D). *Ubx* and *dpp* have the same posterior border of expression (Reuter et al., 1990). Thus *tsh* is primarily expressed in cells that contain *abd-A* protein but also in a few cells containing *Ubx* protein.

The spatial expression of *tsh* in the visceral mesoderm correlates with the requirement for *tsh* function in the midgut. The differences observed between *tsh* and *Antp* protein distribution at the anterior, and between *tsh* and both *Ubx* and *abd-A* at the central region, suggest that *tsh* is not simply responding to any one or combination of the homeotic transcription factors in a cell-autonomous fashion. We show below that *tsh* is activated by the homeotic genes, so either homeotic genes act upon *tsh* at a distance through a signaling molecule, or *tsh* responds both to homeotic genes and to other factors independent of homeotic genes.

Antennapedia, Ultrabithorax, and *abdominal-A* functions are required for expression of *teashirt* in the visceral mesoderm

The anterior domain of *tsh* expression in the visceral mesoderm is largely coincident with cells that express *Antp*, but also includes cells that express neither *Scr* nor *Antp* (Fig. 3A,B). Loss-of-function *Antp* mutations eliminate expression of *tsh* from all of the anterior domain (Fig. 4B). Therefore *Antp* activates *tsh* expression both in cells where *Antp* protein accumulates and, surprisingly, in more anterior cells where *Antp* protein has never been detected. *tsh* expression also follows *Antp* expression in *Ubx* mutant embryos where *Antp* is dere-

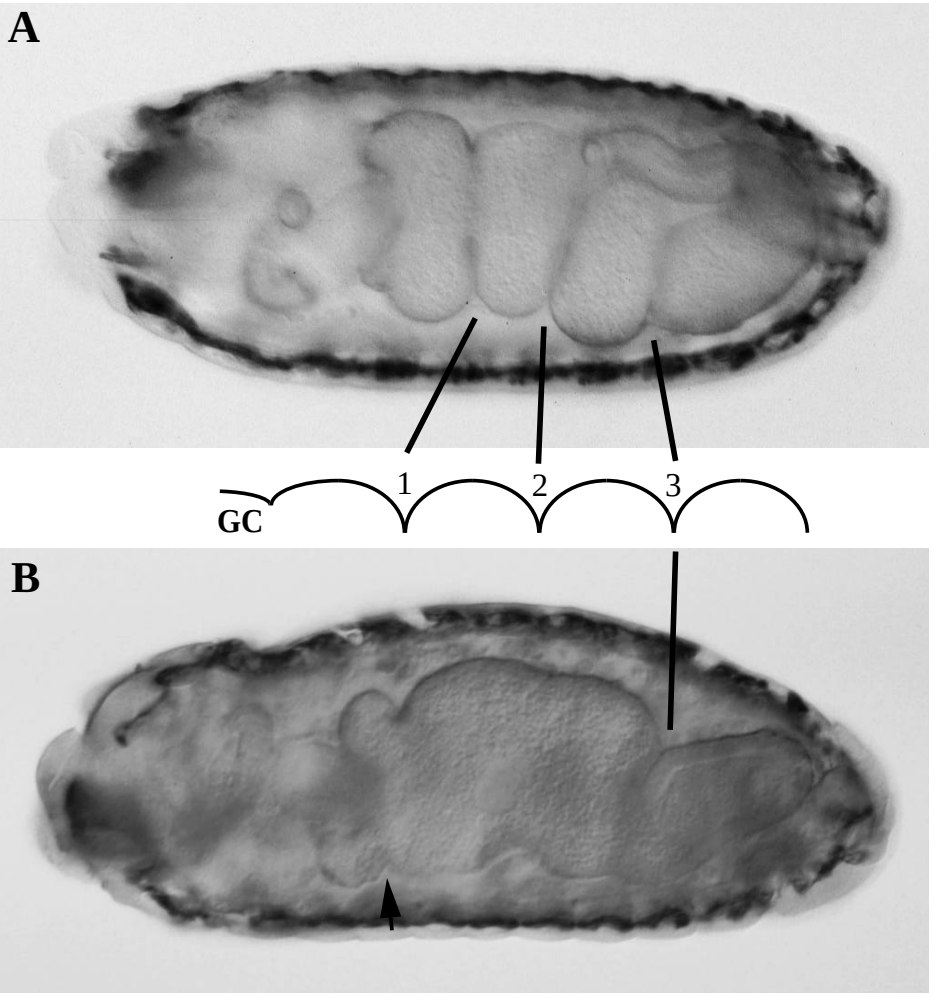


Fig. 1. *teashirt* is required for proper development of the midgut. Stage 16-17 embryos were stained with an antibody to muscle myosin. In wild-type embryos (A), three constrictions (1-3 in diagram) and the budding gastric caeca (GC) are evident. (B) A tsh^{8} embryo does not form the central or anterior constrictions (1 and 2 in diagram). An indentation between the normal position of the anterior constriction and the gastric caeca can be seen (arrow).

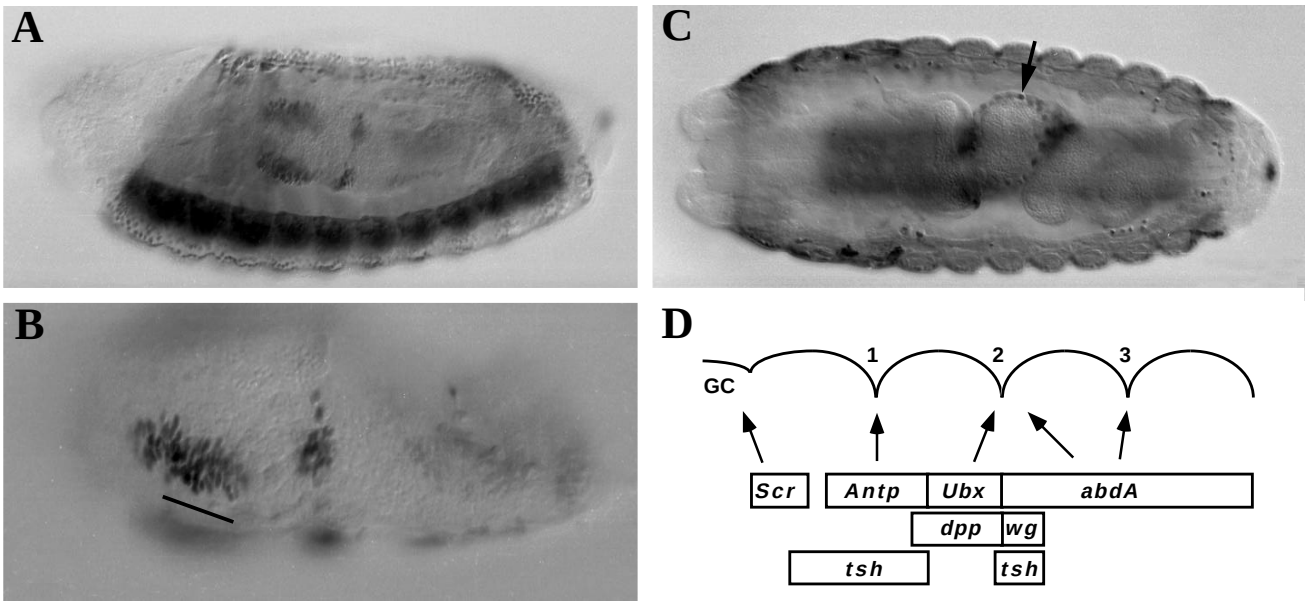


Fig. 2. The *teashirt* protein is expressed in three restricted domains in the midgut. (A) At stage 14, two regions of the visceral mesoderm contain high levels of *tsh* protein. (B) A dissected midgut demonstrates the extent of the two domains. The anterior domain consists of about 14 nuclei overlying the anterior constriction. The bar in B demonstrates the approximate extent of *Antp* expression, based on double-label experiments such as those shown in Fig. 3. A patch of *tsh* protein (about 6 cells) is located just posterior to the future central constriction. Low levels of *tsh* are seen throughout the posterior visceral mesoderm. (C) *tsh* is expressed in the midgut endoderm cells starting during stage 15. The arrow in C points to an endodermal cell expressing *tsh* within the second midgut compartment of a stage 16 embryo. (D) Comparison of *tsh* expression to patterns of homeotic gene and target gene expression in the visceral mesoderm reveals that *tsh* is offset from the homeotic genes.

pressed in more posterior mesoderm cells (Fig. 4C). In wild-type and in *Ubx* mutants, the posterior limit of *Antp* expression is coincident with the posterior limit of *tsh* expression.

The posterior region of *tsh* expression in the visceral mesoderm consists of a small number of cells near the border between the *Ubx*- and *abd-A*-expressing cells. A role for *Ubx* in regulating *tsh* can only be assessed in embryos lacking both *Antp* and *Ubx* functions, since *Antp* is derepressed in *Ubx* mutants and activates *tsh* in more posterior cells (Fig. 4C). In *Antp Ubx* embryos, *tsh* protein is detected in the central visceral mesoderm, but in distinctly fewer cells (Fig. 4D). Therefore, *Ubx* is required for expression of *tsh* in some cells, possibly in the cells that normally contain *Ubx* and *tsh* proteins. Interpretation of *abd-A* mutants is more straightforward since *Antp* is not derepressed (Tremml and Bienz, 1989). Loss-of-function mutations of *abd-A* result in loss of *tsh* protein in all of the cells around the middle constriction (Fig. 5B). The posterior patch of *tsh* expression is regulated by both *Ubx* and *abd-A*.

The combinatorial action of *decapentaplegic* and *wingless* proteins activates *teashirt* expression in the central mesoderm

The movement of the *dpp* and *wg* proteins into the endoderm, and the activation there of *lab* by *dpp*, suggests that these signaling molecules are used in communication between germ layers. One or both secreted proteins may also have a role in the mesoderm and in regulating *tsh*. To assess this possibility it was first necessary to establish more clearly the relative positions of *dpp*-, *wg*-, and *tsh*-expressing cells.

Some cells contain both *wg* and *dpp* protein (Reuter et al., 1990), but it is not known whether this is due to transcription of both genes in the same cells or to movement of one or both secreted proteins into adjacent cells. Here we have used an enhancer trap in the *wg* gene to mark cells where *wg* is expressed. The β-galactosidase protein in the enhancer trap is not secreted and therefore marks only those cells where *wg* is transcribed. The locations of β-galactosidase protein and *dpp* transcripts were compared. *wg-lacZ* and *dpp* mRNA do not accumulate in the same cells, indicating that *wg* and *dpp* are transcribed in non-overlapping domains (Fig. 3E). *tsh* protein is expressed predominantly in the cells posterior to those expressing *dpp* RNA (Fig. 3D). Thus *tsh* expression occurs in cells where *wg* is transcribed and in one to two cells where *dpp* is transcribed.

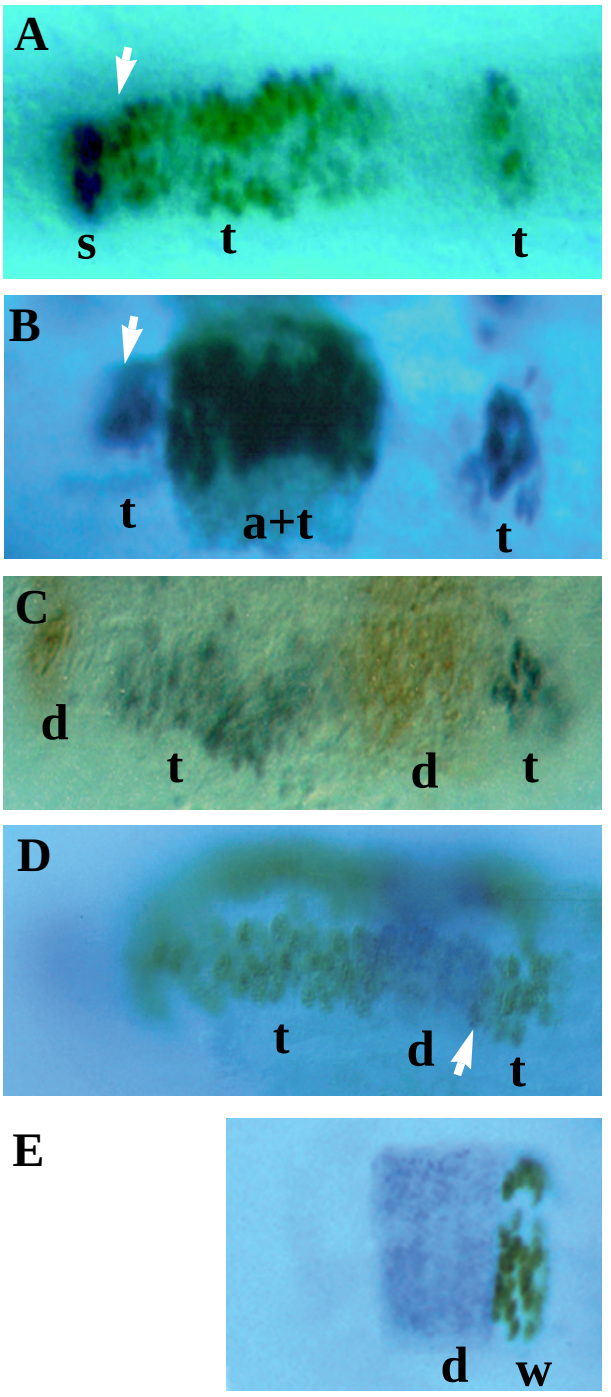
dpp and *wg* both have early functions in embryogenesis. For a clear analysis of their regulation of *tsh* it is necessary to allow the early functions but eliminate the midgut functions. This was accomplished with a *dpp* allele that specifically affects midgut function and with a temperature-sensitive *wg* allele (see Materials and Methods). In embryos that lack *dpp* function in the central region of the midgut, the posterior domain of *tsh* expression is completely absent or significantly reduced (Fig. 5C). Embryos homozygous for a temperature-sensitive *wg* allele were grown at the permissive temperature until gastrulation was complete and then shifted to the restrictive temperature during midgut development (see Materials and Methods). In these embryos the central midgut *tsh* protein is completely absent while anterior expression persists (Fig. 5D). Therefore

both *dpp* and *wg* are required for *tsh* activation in the central region of the midgut. The spatial domain of *tsh* expression in the posterior visceral mesoderm, in addition to the dependence of this expression on *dpp* and *wg*, suggested the possibility that *tsh* may be activated only in cells that encounter sufficient levels of both *dpp* and *wg* proteins.

To examine possible combinatorial actions of *dpp* and *wg* proteins on *tsh*, heat shock-inducible promoters were used to produce one or both signaling proteins in all cells. When *wg* expression is induced in all cells, *tsh* expression is expanded to include the cells between the two normal patches of *tsh* expression (Fig. 6B). Heat shock induction of *dpp* protein expands the *tsh* domain to the posterior (Fig. 6C). The ectopic expression of *tsh* in HS-*dpp* embryos is not continuous; two patches of *tsh* protein are made, the normal central constriction domain and one more posterior. This pattern of *tsh* expression can most easily be explained with reference to ectopic *wg* protein that is observed in HS-*dpp* embryos (Fig. 6E,F). The ectopic *tsh* expression in HS-*dpp* embryos is closely coincident with the ectopic patches of *wg* protein.

The coincidence of ectopic *tsh* expression with both *dpp* and *wg* proteins suggests that both proteins are necessary to induce *tsh* expression in the visceral mesoderm. If *dpp* and *wg* are sufficient to activate *tsh* in the visceral mesoderm, then HS-*dpp* combined with HS-*wg* might result in *tsh* expression throughout the visceral mesoderm. The extent of *tsh* expression is greater in heat-shocked HS-*dpp* HS-*wg* animals than the added effects of HS-*wg* and HS-*dpp* alone, but it does not encompass the entire midgut (Fig. 6D) Low level expression in posterior cells is sometimes detected in HS-*wg* embryos, but never at the levels seen in HS-*dpp* HS-*wg* embryos.

A further test of the requirement for both proteins was performed by removing *dpp* function in the presence of HS-*wg* or by removing *wg* function in the presence of HS-*dpp*. Surprisingly, we find that HS-*wg* can induce the expression of *tsh* even in the absence of *dpp* function in the midgut (Fig. 6G). In contrast, HS-*dpp* cannot activate *tsh* in embryos lacking *wg* function during midgut development (data not shown). Sufficient levels of *wg* protein can activate *tsh* expression without *dpp*, but greater *tsh* activation is observed if *dpp* and *wg* proteins are both ectopically expressed.



DISCUSSION

teashirt, a target of homeotic genes in the midgut

The *tsh* gene is regulated by *Antp*, *Ubx* and *abd-A* in the visceral mesoderm and is required there for proper morphogenesis. Surprisingly few direct or indirect targets of homeotic gene regulation have been identified and characterized. The homeotic genes themselves were the first class of targets of homeotic gene regulation to be identified. The downstream targets found to date include genes encoding secreted molecules (Immerglück et al., 1990; Reuter et al., 1990; Graba

Fig. 3. *tsh* expression in the visceral mesoderm is offset from the homeotic genes. In each panel, midguts dissected from double-labeled embryos are shown to finely map the regions of *tsh* expression within the visceral mesoderm. Expression of *Antp-lacZ* (a), *dpp* (d), *Scr* (s), *tsh* (t) and *wg* (w) is marked in red lower case letters. (A) There is no gap between *tsh* (brown stain) and *Scr* (blue stain) expression in the visceral mesoderm nuclei (arrow). (B) *tsh* protein (blue stain) in the visceral mesoderm nuclei clearly overlaps the *Antp-lacZ* expression (brown stain) in the cytoplasm. *tsh* extends anterior to *Antp* by about 4 cells (arrow). (C) The posterior patch of *tsh* protein (blue staining) overlaps slightly with *dpp* protein (brown stain) in parasegment 7. (D) Because *dpp* protein is secreted, *tsh* expression (brown stain) was compared to *dpp* RNA localization by in situ hybridization (blue stain). Only 1-2 cells contain *tsh* nuclear stain and cytoplasmic *dpp* transcripts (arrow points to a *tsh*-containing nucleus within the *dpp* domain). (E) *dpp* RNA expression was compared to *wg* RNA localization by staining for β-gal protein (brown stain) in a *wg* enhancer trap line. No cells express both *dpp* RNA and *wg* RNA.

et al., 1992; Hursh et al., 1993; Capovilla et al., 1994; Manak et al., 1994), nuclear proteins (Röder et al., 1992; Vachon et al., 1992; Jones and McGinnis, 1993; O'Hara et al., 1993)), a cytoskeletal component (Hinz et al., 1992), and a cell surface molecule (Gould et al., 1990; Gould and White, 1992; reviewed by Andrew and Scott, 1992 and Botas, 1993). Most of the identified target genes, including *tsh*, encode putative regulatory molecules. *tsh* encodes a protein with widely spaced zinc finger motifs (Fasano et al., 1991), and may directly regulate the transcription of genes required for constriction formation. *tsh* is also regulated by homeotic genes in the epidermis and nervous system (Röder et al., 1992).

tsh and the homeotic genes affect epidermal development in

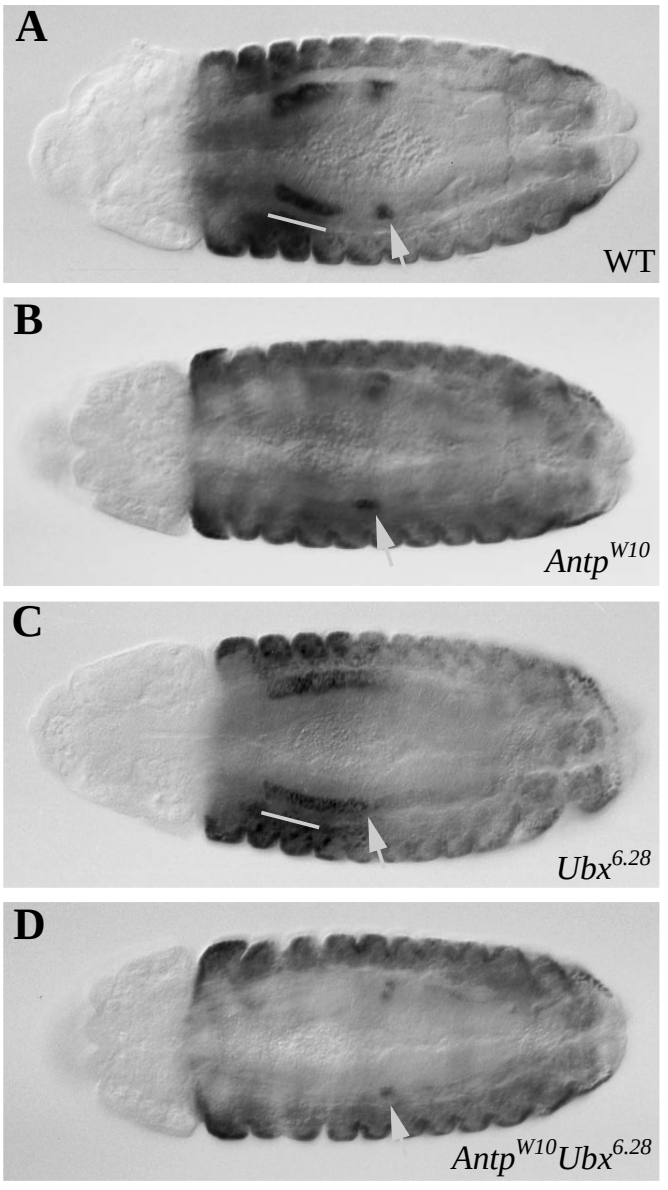


Fig. 4. Expression in the anterior domain of *tsh* protein in the visceral mesoderm reflects the expression of *Antp* protein. (A) Wild-type expression of *tsh* in a stage 13 embryo. A bar marks the normal extent of the anterior domain and an arrow points to the normal central constriction domain. (B) In $Antp^{W10}$ mutant embryos, the entire anterior domain of *tsh* is absent. (C) In $Ubx^{6.28}$ embryos, *tsh* is detected from the normal anterior limit through the posterior (central constriction) limit. *Antp* is derepressed posteriorly to the central constriction in *Ubx* mutants in a manner similar to *tsh* (Tremml and Bienz, 1989; Reuter and Scott, 1990). (D) To assess a role for *Ubx* in *tsh* regulation, $Antp^{W10}Ubx^{6.28}$ embryos were stained for *tsh* protein. $Antp^{W10}Ubx^{6.28}$ embryos retain some *tsh* expression in the central constriction domain. The number of cells in this patch is reduced in these mutants compared to wild type (A vs. D).

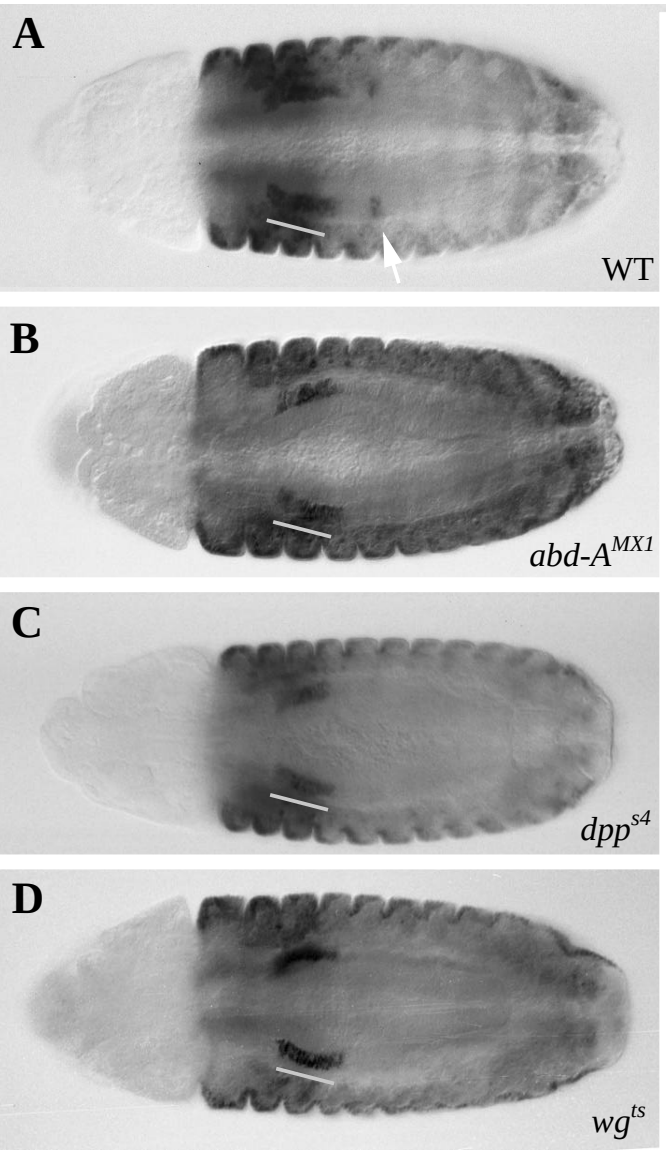


Fig. 5. Expression of *tsh* in the central constriction domain requires the *abd-A*, *dpp*, and *wg* genes. In each panel, a bar marks the normal extent of the anterior domain and an arrow points to the normal central constriction domain when it is present. (A) Wild-type expression of *tsh* in a stage 13 embryo. (B) $abd\text{-}A^{MX1}$ embryos express no *tsh* protein in the central constriction domain. (C) dpp^{s4} embryos lack *dpp* expression in the midgut visceral mesoderm, but sometimes contain a faint posterior patch of *tsh* (not seen in this embryo). This reduction in *tsh* expression is also seen in stage 16 embryos (not shown), when mutants can be identified by the *dpp* midgut phenotype. (D) Embryos containing a wg^{ts} mutation raised at the restrictive temperature during midgut development do not express *tsh* in the central constriction domain.

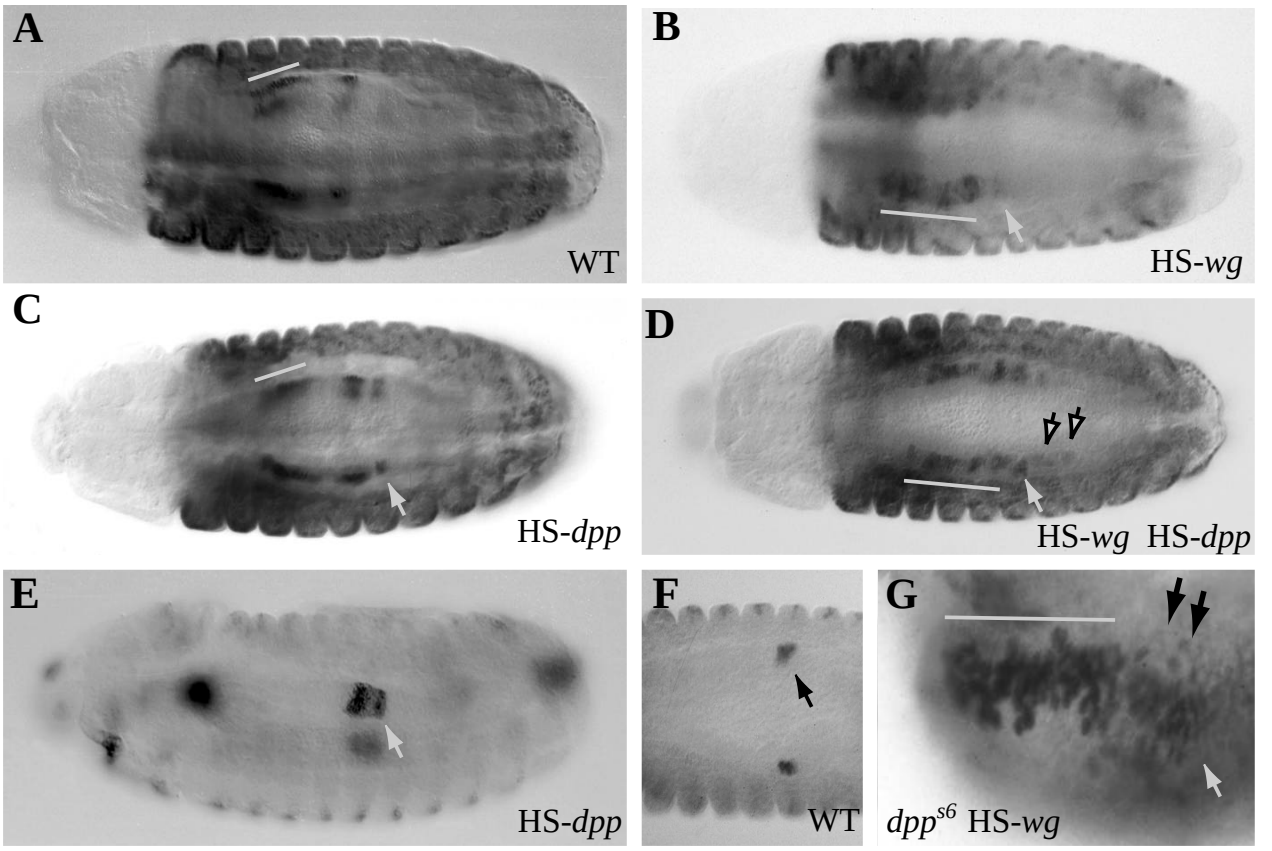


Fig. 6. Ectopic expression of *tsh* is induced by ubiquitously expressing *wg* and/or *dpp* proteins.(A) A wild-type stage 14 embryo stained for *tsh* protein. HS-*dpp* (C, E), HS-*wg* (B), or HS-*dpp* and HS-*wg* (D) transformant embryos were heat shocked, aged for 3 hours, and stained for *tsh* (B-D) or *wg* (E) protein. (C) HS-*dpp* transformant embryos contain an ectopic patch of *tsh* protein posterior to the normal patch. The position of the ectopic patch (arrow) can be determined in later staged embryos when the central constriction begins to form (not shown). (E) The ectopic patch of *tsh* in C probably corresponds to an ectopic patch of *wg* that is detected in HS-*dpp* embryos (arrow). (F) The wild-type expression of *wg* RNA is shown for comparison. (B) *tsh* expression is induced in the cells between the two normal *tsh* domains (bar) in HS-*wg* transformant embryos. (D) In HS-*dpp* HS-*wg* embryos, *tsh* is detected throughout the region containing the two normal *tsh* domains (bar) and in three patches (arrows) posterior to this. One patch of expression (white arrow) likely corresponds to the cells that express *tsh* in HS-*dpp* (compare C and D). Two faint patches (open arrows) of *tsh* are detected posterior to this. Note that some posterior induction of *tsh* can be seen in embryos carrying HS-*wg* alone (white arrow in B). dpp^{s6} HS-*wg* embryos were stained with antibodies to *tsh* and *dpp* (*S6* embryos can be identified because they express *dpp* overlying the gastric caeca, but not in PS7). (G) A dissected midgut from a dpp^{s6} embryo shows continuous *tsh* expression in the anterior midgut, with no detectable *dpp* protein in this region. The normal domains of *tsh* expression are marked by the white bar and arrow. Ectopic *tsh* is marked by black arrows between the two normal domains. Thus the induction of *tsh* in HS-*wg* embryos does not require *dpp*

similar ways, promoting trunk development and preventing head development. In the midgut, *tsh* mutations block the formation of two of the three midgut constrictions, behaving in this respect like *Antp* or *Ubx* loss-of-function mutations. However, *tsh* mutants, in contrast to homeotic mutants, often form ectopic or misplaced 'constrictions', indicating that the cellular machinery necessary for constriction formation can be active in the absence of *tsh* function. *tsh* is therefore likely to control constrictions not by causing synthesis of cytoskeletal components necessary for constrictions but rather by triggering existing machinery in specific locations. *tsh* protein could act in concert with the homeotic proteins to direct expression of farther downstream target genes in an appropriate spatial pattern. *tsh* has previously been demonstrated to act synergistically with homeotic genes in the epidermis to repress head genes (Röder et al., 1992). Like the product of the gene *extradenticle* (Peifer and Wieschaus, 1990; Rauskolb et al., 1993), *tsh* may be a homeotic protein cofactor. *Antp* protein could, for example, activate *tsh* and then both proteins could bind to target genes.

Competence of target genes to respond to regulators

In the simplest situation, a target gene would be expressed in any cell where its activator is found. However, the targets of homeotic genes characterized to date are active in temporal and spatial patterns indicating regulation by tissue-specific and stage-specific factors. A regulator may only activate its target in a subset of the cells in which it is expressed, as in the activation of *wg* by *abd-A*. The presence of inhibitors can prevent a regulator from activating its target, as in the case of *abd-A* interfering with the activation of *dpp* by HS-*Ubx* (Reuter et al.,

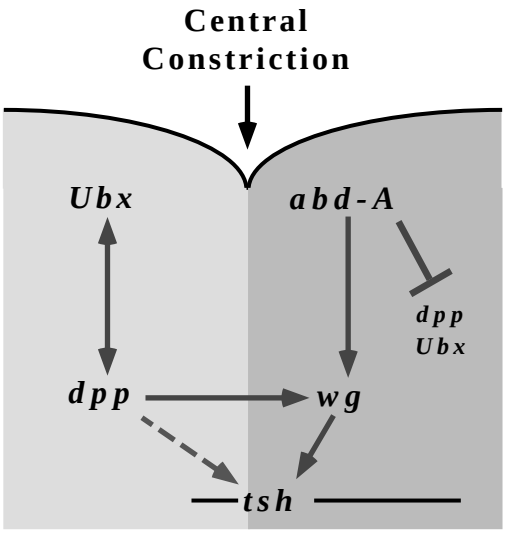


Fig. 7. Summary of gene interactions in the cells surrounding the middle constriction. *Ubx* and *abd-A* are expressed in adjacent cells in PS7 and PS8 respectively. *abd-A* represses the transcription of *Ubx* in the cells posterior to PS7, so no cells express both *Ubx* and *abd-A* (Tremml and Bienz, 1989). *Ubx* activates expression of *dpp* in PS7, while *abd-A* activates expression of *wg* in PS8. *abd-A* prevents *Ubx* from activating *dpp* in the cells posterior to PS7 (Reuter et al., 1990). *wg* and *dpp* are both required for activation of *tsh* in the cells overlying and posterior to the central constriction. *dpp* mutant embryos have reduced levels of *wg* protein (Immergluck et al., 1990). Although *dpp* influences *tsh* expression through the activation of *wg*, it also enhances the activation of *tsh* by ubiquitous *wg* production (shown as a dotted arrow).

1990). There may also be coactivators, or corepressors, needed for a regulator to act, as in salivary gland induction by *Scr* only in cells not making *tsh* (Andrew et al., 1994).

The ectopic induction of *tsh* by ubiquitous expression of *wg* and *dpp* occurs in the posterior in two patches, but not in all of the posterior visceral mesoderm. Thus only some of the posterior visceral mesoderm cells are competent to express *tsh* in response to *wg* and *dpp*. Perhaps not all posterior cells produce other factors necessary for *tsh* induction by the *wg* and *dpp* signals, or some cells require a higher level of *wg* or *dpp* protein than can be obtained by our methods. The restricted expression or differential levels of receptors for the *wg* and *dpp* signals, for example, would limit ectopic activation of *tsh* in response to the signals. In the fly eye, the R3, R4, R1, and R6 photoreceptor precursors are prevented from adopting the R7 cell fate in response to the signal from R8 cells (Hiromi et al., 1993). Expression of the *seven-up* gene, a member of the steroid receptor superfamily, in the R3/R4/R1/R6 cells is necessary to limit the action of the *bride of sevenless* signal on these cells (Mlodzik et al., 1990). Similarly, the limited response of posterior visceral mesoderm cells to the *wg* and *dpp* signals might be limited by the expression of a negative regulator in some cells.

Non-autonomous effects of homeotic genes

In the fly epidermis, homeotic genes act autonomously: in genetically mosaic flies lacking homeotic function in some cells, cells behave according to their genotypes and ignore what neighboring cells are doing (Garcia-Bellido and Lewis, 1976; Struhl and Brower, 1982). The present study provides examples of non-autonomous effects of homeotic mutations in the midgut. First, the *tsh* expression, which is eliminated in *Antp* mutants, extends beyond the domain where *Antp* protein is detected. The discrepancy between the anterior limit of *Antp* expression and the domain of *Antp* influence on *tsh* expression can most easily be explained by the action of diffusible factors whose synthesis or activity is regulated. The existence of a signal from *Antp*-expressing cells to *Scr*-expressing cells has been previously postulated (Reuter and Scott, 1990). Although no midgut cells activate both of these homeotic genes, and in fact there is a small gap between cells that contain *Scr* protein and cells that contain *Antp* protein, homozygous *Antp* mutants produce lower amounts of *Scr* protein in the anterior midgut. Perhaps *tsh* is activated by the same factor(s) through which *Antp* activates *Scr* expression at a distance. *tsh* is inducible, in the epidermis and in the anterior midgut visceral mesoderm, by *Antp* protein controlled by a heat shock promoter (Röder et al., 1992; Zeng et al., 1993). Together the results indicate that *Antp* controls *tsh*, but do not prove a direct interaction. In cells where both *Antp* and *tsh* are expressed, the interaction could be direct. However, cells that make *tsh* but not *Antp* protein must receive a *tsh*-inducing signal from more posterior cells that contain *Antp* protein. The range of movement of the postulated signaling molecule might determine the anterior border of *tsh* expression.

A second example of non-autonomy comes from the effects of *abd-A* on *tsh*. *tsh* is expressed in a few cells that contain *Ubx* protein and not *abd-A* protein. This observation is most easily explained by the secretion of *wg* protein from *abd-A*-expressing cells and its short range movement to or into more anterior mesoderm cells. High-level *tsh* expression is detected in only the anterior portion of the *abd-A* protein domain of the mesoderm. Induction of *tsh* by *wg* may also explain the limited expression of *tsh* within the *abd-A* domain. Regulation of secreted factors by homeotic genes provides a simple mechanism for obtaining patterns of expression which are not a simple readout of the homeotic expression patterns.

Integration of Wnt and TGFβ class signals

Integration of *dpp* and *wg* signals has been suggested to occur in three cases: in the regulation of *Distal-less* (*Dll*) in the thoracic imaginal primordia (Cohen et al., 1993), in regulation of *aristaless* (*al*) in the imaginal discs (Campbell et al., 1993), and in *tsh* regulation in the midgut. We show here that *tsh* protein is detected near the intersection of *dpp* and *wg* expression domains in the visceral mesoderm and that expression of *tsh* requires both *dpp* and *wg*. The *wg*-dependent activation of *al* and *Dll* occurs in cells near the intersection of *dpp* and *wg* expression domains. The activation of *Dll* by *wg*, in the embryonic limb primordia, occurs in cells near *dpp*-expressing cells. Ectopic induction of *wg* in imaginal discs results in ectopic *al* in regions where *dpp* is also present, which makes the involvement of *dpp* or another regulator expressed in a similar pattern likely. However, the role of *dpp* in induction of *al* and *Dll* has not been tested and remains hypothetical. Perhaps the *dpp* and *wg* signals are integrated by cells near the intersection of the two signals.

We have further tested the possible collaboration of *dpp* and

wg in *tsh* activation in the midgut. *dpp* function is necessary for *tsh* activation. However, *dpp* mutant embryos have diminished *wg* expression (Immergluck et al., 1990), which could explain the loss of *tsh* expression. *wg* protein provided ubiquitously using a heat-inducible promoter activates *tsh* expression in the same cells in wild-type or *dpp* mutant embryos. This result suggests that *dpp* may only be necessary to maintain *wg* expression. However, the combined action of HS-*dpp* and HS-*wg* results in strong *tsh* induction in more cells than with either HS-*wg* or HS-*dpp* alone. In the simplest interpretation of these results *wg* is sufficient to activate *tsh* expression in the absence of *dpp*. *dpp* protein might enhance *tsh* induction by *wg* by activating the expression of genes necessary for *wg* signaling, or by stabilizing *wg* or any of its signal transduction machinery (Fig. 7). An alternative explanation is the existance of another protein, perhaps of the TGFβ class, which can substitute for *dpp* protein. In *dpp* mutants ectopic *wg* protein would be capable of activating ectopic *tsh* to an extent limited by the postulated unknown protein. The provision of ectopic *dpp* protein would remove this limitation and lead to higher *tsh* levels in HS-*wg*; HS-*dpp* than in HS-*wg* embryos. If the integration of TGFβ and Wnt class signals is a common developmental mechanism, identification of additional genes in this pathway in the midgut will help clarify this interaction.

We thank Danny Brower, F. Michael Hoffmann, Dan Kiehart and Roel Nusse for gifts of antibodies, and Kathy Matthews and her colleagues at the Indiana Stock Center for continuing great help with fly stocks. We are grateful to David Bilder, David Eisenmann, David Kingsley, and one non-David--Roel Nusse--for critical readings of the manuscript. We thank Ron Blackman, Richard Padgett and Bill Gelbart for making available the HS-*dpp* construct prior to publication, and Barbara Hill for excellent technical assistance. This work was supported by grant no. HD-18163 to M. P. S. from the National Institutes of Health. M. P. S. is an Investigator of the Howard Hughes Medical Institute.

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(Accepted 7 July 1994)