Homeotic genes regulate the spatial expression of putative growth factors in the visceral mesoderm of *Drosophila* embryos

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Summary

During *Drosophila* embryogenesis homeotic genes control the developmental diversification of body structures. The genes probably coordinate the expression of as yet unidentified target genes that carry out cellular differentiation processes. At least four homeotic genes expressed in the visceral mesoderm are required for midgut morphogenesis. In addition, two growth factor homologs are expressed in specific regions of the visceral mesoderm surrounding the midgut epithelium. One of these, *decapentaplegic* (*dpp*), is a member of the transforming growth factor β (TGF-β) family; the other, *wingless* (*wg*), is a relative of the mammalian proto-oncogene *int-1*. Here we show that the spatially restricted expression of *dpp* in the visceral mesoderm is regulated by the homeotic genes *Ubx* and *abd-A*. *Ubx* is required for the expression of *dpp* while *abd-A* represses *dpp*. One consequence of *dpp* expression is the induction of *labial* (*lab*) in the underlying endoderm cells. In addition, *abd-A* function is required for the expression of *wg* in the visceral mesoderm posterior to the *dpp*-expressing cells. The two growth factor genes therefore are excellent candidates for target genes that are directly regulated by the homeotic genes.

Key words: Ultrabithorax, decapentaplegic, labial, abdominal-A, wingless, visceral mesoderm, homeotic, growth factor, *Drosophila*.

Introduction

Mutations in *Drosophila* homeotic genes transform one part of the fly into another. The best studied phenotypes involve changes in the formation of the appendages and cuticle of the adults and of the larval cuticle, but the genes affect internal structures as well. We have used the development of the midgut to analyze the roles of homeotic genes in forming a normal embryo, to characterize interactions among the homeotic genes, and to identify genes that are regulated by the homeotic genes.


The mechanisms by which homeotic genes influence morphogenesis remain largely unknown. All of the ANT-C and BX-C homeotic genes encode proteins with homeodomains, sequence-specific DNA-binding domains (reviewed in Scott *et al.* 1989), and several of the protein products of the homeotic genes have been shown to function as transcription factors (Jaynes and O'Farrell, 1988; Han *et al.* 1989; Driever and Nüsslein-Volhard, 1989; Hanes and Brent, 1989; Krasnow *et al.* 1989; Struhl *et al.* 1989; Winslow *et al.* 1989). These observations strongly suggest that the genes control
morphology by coordinating the spatial and temporal transcription patterns of target or 'realizator' genes (Garcia-Bellido, 1977). In the epidermis, the nervous system, and the visceral mesoderm, cross-regulatory interactions have been observed among the homeotic genes; thus the first target genes to have been identified are homeotic genes (Hafen et al. 1984; Struhl and White, 1985; Carroll et al. 1986; Casanova and White, 1989). In addition some of the homeotic genes have been found to auto-regulate, including one, Ubx, that activates its own expression in the visceral mesoderm (Bienz and Tremml, 1988). There must, however, be other genes that are controlled by homeotic genes but are more directly linked to the processes of cell differentiation and morphogenesis. In an attempt to identify such target genes we have looked for genes that are transcribed in restricted regions of the embryo overlapping with domains of homeotic gene expression. Two genes, wingless (wg) and decapentaplegic (dpp), that encode putative growth factors are expressed in the visceral mesoderm of the developing midgut in patterns that suggest they may be regulated by homeotic genes. Several of the homeotic genes, including lab, Scr, Antp, Ubx, and abd-A, are expressed in discrete regions in the developing midgut. The midgut is made up of two cell layers: an inner layer of large polypliod endoderm cells ensaced in a sheath of smaller, eupaloid visceral mesoderm cells. The four homeotic genes Scr, Antp, Ubx, and abd-A are expressed in non-overlapping domains in the visceral mesoderm cells in an order along the anterior–posterior axis similar to that in the epidermis, i.e. Scr is active in the most anterior region of the mesoderm, then Antp, then Ubx, and abd-A in the most posterior domain (LeMotte et al. 1989; Tremml and Bienz, 1989; Reuter and Scott, 1990). Defects in gut morphogenesis are seen when any of these four homeotic genes is defective. The morphological alterations seen in the gut in the homeotic mutants generally correspond well with where the products are found in wild-type embryos. The gastric caeca, four tentacle-like protrusions of the midgut at its anterior end, do not form if Scr function is lacking (Reuter and Scott, 1990). Constrictions that divide the gut into sections normally form at three positions along the anterior–posterior axis of the gut tube, and each constriction is dependent on homeotic gene function (Tremml and Bienz, 1989; Reuter and Scott, 1990). The anterior constriction that underlies the mesoderm cells that express Antp does not form in mutant embryos that lack Antp function. The middle constriction that normally forms near the interface between Ubx and abd-A expression fails to develop in either Ubx or abd-A mutants, and the posterior constriction that underlies abd-A expression does not form if abd-A function is lacking. There is evidence that these morphological features are formed by the imposition of the structure upon the endoderm by the mesoderm (Reuter and Scott, 1990); however, it is possible that both cell layers are active participants in the formation of the constrictions. The lab gene is expressed only in a subset of the endoderm cells, and defects in the formation of the second midgut constriction have been reported in lab mutants (Immerglick et al. 1990). Thus, a key element of the control of differentiation by homeotic genes in the midgut, as well as in the epidermis, is the differential spatial expression pattern of each gene. The sequencing of wg revealed that its product is closely related to the product of the mammalian gene int-1 (Rijswijk et al. 1987). The wg gene is expressed in early embryos in stripes corresponding to the posterior part of each parasegment (PS; Baker, 1987) and is one of several Drosophila genes involved in establishing polarity of the embryo (Ingham, 1988). The protein is made in about one-quarter of the cells in each segment primordium, where the RNA is seen, and then some of the protein appears to move into adjacent cells (van den Heuvel et al. 1989). Studies of mosaic adult flies in which some of the cells lack all wg function while other surrounding cells have one functional copy of the gene have shown that mutant cells can be rescued by the neighboring wild-type cells (Morata and Lawrence, 1977). These observations, as well as the change in the polarity of cell structures in parts of segments in wg mutant embryos where its RNA is not transcribed, suggest that the protein is involved in cell–cell communication processes. The wg products are also detected in the developing midgut, in a narrow band of visceral mesoderm cells (van den Heuvel et al. 1989) suggesting that it also has a function in the differentiation of the gut. The location of wg products in the midgut led us to examine whether wg is regulated by homeotic genes of the BX-C. We demonstrate that wg transcription is activated by abd-A, but only in a discrete subset of the visceral mesoderm cells where abd-A is expressed, indicating that abd-A is necessary, but not sufficient, for wg expression. The decapentaplegic (dpp) gene was first recognized for its effects on the development of adult structures derived from imaginal discs (Spencer et al. 1982; St Johnston et al. 1990), and was subsequently found to be involved in other processes such as the development of proper dorsal–ventral patterning (Irish and Gelbart, 1987). It encodes a product that is a member of the TGF-β family of growth factors (Padgett et al. 1987). The protein, synthesized in cultured cells, has been found to be cleaved and secreted, suggesting that it is involved in carrying information between cells (Panganiban et al. 1990a). The pattern of expression of the gene is complex, in keeping with the gene's functions in many different cells and tissues (St Johnston and Gelbart, 1987; Masuccio et al. 1990; Jackson and Hoffmann, in preparation). One of the places that dpp RNA and protein is found is in the developing midgut. The RNA is detectable only in the visceral mesoderm cells in particular positions, while the protein is found in the same cells as the RNA and additionally in some of the underlying endoderm cells (Panganiban et al. 1990b). We have explored the relationship between Ubx and dpp further by studying the induction of dpp by ectopic expression of Ubx. We demonstrate that Ubx induces the expression of dpp in visceral mesoderm cells of the
anterior half of the midgut. Induction of dpp by Ubx is restricted to the mesodermal cells suggesting that Ubx must act together with other factor(s) limited to the visceral mesoderm to induce dpp, or that the action of Ubx upon dpp is blocked in all tissues but the visceral mesoderm. dpp expression in the posterior of the midgut is inhibited by abd-A, even in the presence of Ubx protein. The action of abd-A upon dpp is therefore not due to repression of Ubx by abd-A. The ectopic production of Ubx protein, and hence dpp protein, in the visceral mesoderm leads to ectopic expression of lab in many of the underlying endodermal cells.

Materials and methods

Fly strains

Stocks are described in Lindsey and Grell (1968) and additional references are as follows. The mutant stocks used were st Ubx628 ec abd-TM1 ([Kerridge and Morata, 1982], no detectable Ubx protein due to the deletion of 32bp in the S' exon which causes a frameshift at codon 27 and premature stop of translation (Beauchy et al. 1985; Weinzeller et al. 1987]), abd-A/TM6B ([Sanchez-Herrero et al. 1985] no detectable abd-A protein [Karch et al. 1986]), Df(3R)hs210/TM1 [deficient for the Ubx transcription unit (Lewis, 1978; Bender et al. 1983)], Df(3R)Ubx095 gli e/Dp (33) F5, Sb [Lewis, 1978], deficient for the Ubx and abd-A transcription units (Karch et al. 1986), Df(3R)F9/Dp(3;3)F5, Sb [Lewis, 1978], deficient for the complete bithorax complex (Karch et al. 1985), Sce17 Ki/TM3 Sb and Sce0 cu p/TM3 Sb [Wakimoto and Kaufman, 1984], no detectable Ser protein (Riley et al. 1987), Antp107 red e/TM3, Sb Ser [Wakimoto and Kaufman, 1984], no detectable Antp protein (Carroll et al. 1986), Df(3R)Antp107+ peri 3cu e y1/TM3, Sb Ser [Deficiency which takes out the Antp protein-coding region (Struhl, 1981; Gubser et al. 1983)].

The transformant line hs/Ubx which carries an Ubx cDNA under the control of the hsp70 promoter on a rosy' chromosome was provided by G. Struhl (Gonzalez-Reyes et al. 1990); a similar construct was provided by R. Mann and D. Hogness. The transformant line HSU-109 which carries a heat shock promoter-controlled Ubx cDNA on a Df(3R)Ubx095 chromosome was provided by A. Gonzalez-Reyes (Gonzalez-Reyes et al. 1990).

Antibodies

The origins of the antibodies used in this study are as follows. The anti-dpp antibody was generated as described by Panganiban et al. (1990a). The hydridoma line FP2.38 (anti-Ubx) was kindly provided by White and Wilcox (1984), the hydridoma line 4C3 (anti-Antp) by D. Brower (J. Condie and D. Brower, unpublished results) and the hydridoma line 6H4 (anti-Ser) by Glickman and Brower (1988). The anti-lab antibody was kindly provided by T. Kaufman (Diederich et al. 1989), the anti-abd-A antibody by W. Bender (Karch et al. 1990) and the anti-wingless antibody by R. Nussel (van den Heuvel et al. 1988). The antibodies were used as hydridoma supernatants diluted 1:10 (anti-Ubx) or 1:3 (anti-Ser and anti-Antp) in PBT (see below) or as affinity-purified antibodies diluted 1:200 (anti-abd-A), 1:500 (anti-wingless) or to 1 μg ml−1 (anti-dpp) in PBT.

Heat-shock expression of the Ultrathorax protein

Eggs were collected for 3h or overnight at 25°C and the embryos were heat-shocked at 37°C for two times 20min with a 20min period at 18°C in between. Subsequently, the embryos were kept for three hours or the time period given in the text at 25°C for recovery.

Immunostaining of embryos

Embryos were fixed as described by Karr and Alberts (1986) and were immunostained as described by Reuter and Scott (1990). Staging was according to Campos-Ortega and Hartenstein, 1985. Antibodies raised in rabbits were immuno-localized using biotinylated secondary antibodies (Vector Lab.) and the ELITE peroxidase detection kit from Vector Lab. For the double label immunostaining the procedure was modified as follows. Fixed and PBT-saturated embryos were first simultaneously incubated with two primary antibodies from different species, for example anti-Ubx and anti-dpp, and then, after extensive washing, with a dilution of goat anti-mouse IgG peroxidase conjugate (Biorad) and of biotinylated goat anti-rabbit IgG (Vector Lab.). The embryos were washed again, and the bound murine antibodies were detected by oxidation of diaminobenzidine (DAB) in the presence of nickel and cobalt ions which gives a dark gray stain (Lawrence et al. 1987). The DAB solution was thoroughly removed before peroxidase activity was destroyed by the addition of hydrogen peroxide to 3%. Subsequently, the rabbit antibody was detected after incubation with an avidin-peroxidase complex (Vector Lab) by the oxidation of DAB in the absence of heavy metal ions which gives a brown stain.

Results

Ultrathorax expression is required for the expression of decapentaplegic in the visceral mesoderm

dpp is expressed in three regions of the visceral mesoderm surrounding the midgut: anteriorly, in the mesoderm covering the developing gastric caeca, centrally, close to the position where the middle constriction of the midgut forms, and posteriorly, at the site of the third midgut constriction (St Johnston and Gelbart, 1987; Panganiban et al. 1990b; Fig. 1A). The third constriction expression is usually not detected by the dpp antibody. The homeotic gene Ubx was also found to be expressed in the central region of the visceral mesoderm, in PS 7 (White and Wilcox, 1984; Bienz and Treisman, 1988). Indeed, when dpp and Ubx protein are simultaneously detected in embryos by a double label experiment, they are expressed in overlapping domains (Fig. 1C). The posterior borders of their expression coincide precisely; anteriorly dpp protein is detected half a segmentation anterior of the Ubx protein.

Because Ubx is known to regulate transcription (Krasnow et al. 1989), the overlapping domains of expression of dpp and Ubx led us to investigate whether Ubx controls dpp. In embryos that lack Ubx protein, dpp protein is virtually absent from the visceral mesoderm in PS 6 and 7 (Fig. 1B). Only a faint band of dpp protein remains detectable around the midgut. This is located in the posterior part of the normal dpp expression domain (Fig. 1B), directly adjacent to the region where wg and abd-A are expressed in the visceral mesoderm (Fig. 1D).
The second domain of \textit{dpp} expression in the visceral mesoderm (Fig. 1A), in cells that later cover the budding gastric caecae, is less than half a parasegment wide and is located anterior to the domain of \textit{Scr} protein expression (data not shown). \textit{dpp} expression here is not influenced in embryos by the lack of \textit{Ubx} function (Fig. 1B) and also persists in embryos mutant for either of the homeotic genes \textit{Scr} or \textit{Antp}, both of which are normally expressed in the visceral mesoderm (data not shown).

\textbf{Ultrabithorax expression is sufficient for the expression of decapentaplegic in the visceral mesoderm}

To more conclusively test whether or not \textit{dpp} is activated by \textit{Ubx} we examined the expression of \textit{dpp} in embryos that carry an \textit{Ubx} cDNA under the control of the heat shock hsp70 promoter (HSU) (Mann and Hogness, 1990; Gonzalez-Reyes et al., 1990). One hour after a heat shock \textit{Ubx} protein can be detected in every cell nucleus of the embryo at high levels (data not shown). In these embryos \textit{dpp} is ectopically expressed in the visceral mesoderm from the anterior end of the midgut to PS 7 (Fig. 2C). In other tissues, the expression of \textit{dpp} protein changes little if at all.

In embryos homozygous for mutations in the \textit{abd-A} gene, \textit{Ubx} is expressed at high levels in posterior regions where \textit{abd-A} would normally be expressed (Struhl and White, 1985; Bienz and Trempml, 1988). In such embryos \textit{dpp} is also ectopically expressed in the posterior visceral mesoderm, but not in other tissues (Fig. 2B; Immergult et al., 1990; Panganiban et al., 1990b). Embryos that lack both \textit{Ubx} and \textit{abd-A} expression (\textit{Df(3R)P9} or the entire bithorax complex) express \textit{dpp} in a pattern that is different from any \textit{dpp} pattern seen in wild-type embryos (Fig. 2D). The \textit{dpp} expression that normally overlaps with \textit{Ubx} is gone completely, but \textit{dpp} protein is found in a segmentally modulated pattern, in five faint stripes, in the region of the visceral mesoderm where \textit{abd-A} is normally expressed. This observation indicates that \textit{abd-A} negatively regulates \textit{dpp} in the absence of \textit{Ubx}. \textit{dpp} expression in the posterior part of the midgut that is not dependent on the homeotic genes \textit{Ubx} and \textit{abd-A} might result from influences of earlier acting genes such as segmentation genes.

\textbf{abdominal-A negatively regulates decapentaplegic in the visceral mesoderm, but not via Ultrabithorax.}

In spite of the ubiquitous nuclear \textit{Ubx} protein in the HSU embryos after a heat shock treatment, \textit{dpp} protein is not found in the posterior part of the visceral mesoderm where the \textit{abd-A} protein is present (Fig. 2C). The posterior border of \textit{dpp} expression is not changed by the heat shock-driven ectopic \textit{Ubx} expression (compare embryos in Figs 2A and C). In order to test whether \textit{abd-A} is the negative regulator that prevents the ectopic \textit{Ubx} from activating \textit{dpp}, \textit{dpp} expression in HSU embryos that lack \textit{abd-A} function was examined. For this purpose embryos were used that carry HSU on a third chromosome that lacks both \textit{Ubx} and \textit{abd-A} function (\textit{Df(3R)Ubx\textsuperscript{109}}). This chromosome will hereafter be referred to as HSU-109 (Gonzalez-Reyes et al., 1990). The absence of the endogenous \textit{Ubx} gene is required for the experiment because otherwise \textit{dpp} protein would be ectopically expressed in the posterior part of the visceral mesoderm even without the heat shock-driven expression of \textit{Ubx}, just due to the derepression of \textit{Ubx} in \textit{abd-A} mutant embryos (Fig. 2B). The genotype of the embryos that have \textit{dpp} protein all through the midgut was established by using an anti-\textit{abd-A} antibody (Karch et al., 1990) to show that they lack the \textit{abd-A} protein (data not shown).

About one-quarter of the heat-shocked embryos obtained from HSU-109 heterozygous parents, those that lack \textit{Ubx} and \textit{abd-A}, have ectopic \textit{dpp} protein throughout the entire visceral mesoderm of the midgut (Fig. 2E). Therefore the removal of a single gene function, \textit{abd-A}, allows HSU, or the ectopic \textit{Ubx} in \textit{abd-A} mutants, to induce \textit{dpp} expression in the posterior visceral mesoderm. Consequently, \textit{dpp} is a likely candidate for a target gene directly negatively regulated by \textit{abd-A}.

\textbf{Induction of labial requires direct contact with visceral mesoderm cells during early midgut development.}

The study described in the accompanying paper (Panganiban et al. 1990b) shows that the endoderm receives \textit{dpp} as a signal from the mesoderm. \textit{dpp} protein can be detected at the apical side of the endoderm; it moves from one germ layer to the other, and it is required for \textit{labial (lab)} expression in the central part of the midgut endoderm (Panganiban et al., 1990b). A close inspection of the \textit{lab} expression in wild-type embryos revealed some interesting details of the signal transduction from visceral mesoderm to endoderm. In stage 13 embryos the endoderm forms a multi-layered cluster of cells beneath the expression domain of \textit{Ubx} and \textit{dpp}. Of these cells only the outer layer, which is in direct contact with the visceral mesoderm, accumulates \textit{lab} protein; the adjacent cells of the inner layers do not (Fig. 3A,F). Later, from stage 15 on, the inner endodermal cells become integrated into the single cell layer of the endoderm (Fig. 3E; diagrammed in Fig. 3F). In stage 17 endodermal cells within the epithelial sheet that do not express \textit{lab} can be clearly distinguished from cells that do express it (Fig. 3B,D,E). Thus even though at stages 15–17 \textit{dpp} protein is made and is moving between germ layers, some cells still do not produce \textit{lab} protein in response.

\textbf{Ectopic decapentaplegic protein activates labial expression in the endoderm.}

Here we provide further evidence that \textit{dpp} protein spatially controls \textit{lab} expression in the endoderm. In HSU embryos \textit{lab} is detectable three hours after heat shock in most of the anterior endoderm of the midgut, underlying the cells where \textit{dpp} is ectopically activated in the visceral mesoderm by \textit{Ubx}. (Fig. 4A). The ectopic \textit{lab} expression is transient; five hours after the heat shock the amount of \textit{lab} protein in the anterior part of the midgut has decreased (Fig. 4B). Even in...
Fig. 1. Expression of decapentaplegic protein in the visceral mesoderm of wild-type and Ubx null mutant embryos. Optical sections of whole mount preparations viewed by differential interference contrast microscopy. (A,B) dpp protein in the visceral mesoderm of wild-type (A) or homozygous UbxΔ28 embryos (B) at stage 13; shown in a dorsolateral view (between arrowheads: dpp expression domains; arrows: endodermal cell cluster; ed: endoderm; vm: visceral mesoderm; hg: hindgut). Ubx and dpp protein (C) or Ubx, dpp, and wg proteins (D) have been simultaneously detected in wild-type or homozygous Ubx6.28 embryos (D) by double-label immunolocalization. Ubx protein is visualized in black and indicated between arrowheads in the visceral mesoderm. dpp (C,D) and wg (D) protein are visualized in brown and pointed out between arrows. No Ubx protein is visible in D due to the Ubx mutation.

Fig. 2. Ectopic expression of decapentaplegic in various mutant and transformant embryos. dpp protein in the visceral mesoderm of (A) a wild-type embryo, (B) an abd-AΔ91, and (D) a Df(3R)Ubx109 homozygous mutant embryo or (C) a HSU and (E) a HSU-109 embryo three hours after a heat shock. In HSU-109/TM1 progeny about three-quarters of the embryos in the collection make Ubx protein ubiquitously after a heat shock treatment; the others are TM1/TM1. Of those carrying HSU about two-thirds have ectopic dpp expression similar to that of the HSU embryo in C. About one-third of the embryos, presumably the HSU-109 embryos that lack Ubx and abd-A, have ectopic dpp protein throughout the entire visceral mesoderm of the midgut as in E. The homozygous mutant embryos have been identified among their siblings due to their lack of Ubx (D) or of abd-A (B,E) protein expression in double staining experiments. The arrows point to the cluster of large endodermal cells. The arrowheads (A,C) indicate the approximate anterior end of the visceral mesoderm surrounding the developing midgut.
Fig. 3. Expression of *labial* protein in the endodermal cells of embryos after germ band retraction. *lab* protein in the endoderm of (A,C) a stage 14 and (B,D,E) a stage 17 embryo. (C) A close view of the middle region of the midgut of a stage 14 embryo (cells in the same region delimited by arrowheads in panel A, but from a different embryo). Arrows in C point to the nuclei of the large endodermal cells that are located beneath the *lab* expressing cells and do not express *lab*. The large cells have a remarkably pronounced nucleoli (yk: yolk; ed: endoderm; vnc: ventral nerve chord). (D) Close-tangential view of a stage 17 embryo similar to the one in panel B. Cells of the *lab*-expressing midgut loop viewed with a low depth of field. Arrows indicate non-*lab*-expressing cells that probably had been part of the endodermal cluster and are now integrated into the single-layered endoderm. (E) Optical section of a similar midgut loop. Arrow has same meaning as in D. (F) Schematic summary of the proposed integration of the cluster of endodermal cells ([][])) into the midgut epithelium within the *lab* expressing domain (●●●).
Fig. 4. Expression of *labial* protein in HSU embryos after heat shock. Expression of *lab* protein in a HSU embryo of approximately stage 13 three hours after heat shock (A) and a HSU embryo of approximately stage 16 five hours after heat shock (B). The arrowheads indicate the relatively sharp anterior border of the ectopic *lab* domain within the midgut endoderm. (C) High magnification view of a HSU embryo three hours after heat shock at approximately stage 13. The arrows point to the cells of an endodermal cluster which, in the heat-shocked embryos as in normal embryos, do not express *lab* protein. (D) Lateral view of the anterior midgut of a stage 14 HSU embryo after heat shock. (E,F) Close lateral view in two focal planes of the midgut of a stage 14 HSU embryo after heat shock which reveals the large endodermal cells that do not express *lab* (F; arrows) beneath the endodermal cells that contain large amounts of *lab* protein.
Fig. 5. *wingless* protein expression in the visceral mesoderm depends on *abdominal-A* function. *wg* protein in the visceral mesoderm of (A) a wild-type embryo, (B) a *Ubx*<sup>2-28</sup>, (C) a *abd-A*<sup>Ubx</sup>, and (D) a *Df(3R)P99* homozygous mutant embryo. The *wg*-expressing visceral mesoderm cells are indicated by arrowheads. The arrows point at the endodermal clusters. While the *wg* expression in the visceral mesoderm of the *Ubx*<sup>2-28</sup> embryo is indistinguishable from the wild-type pattern, *wg* protein is absent from this tissue in all mutant embryos that lack *abd-A* function (C,D). (E) In embryos deficient for *abd-A*, *Ubx*, *pbx*, and *txd* functions, i.e. *Df(3R)bxd<sup>100</sup>* homozygotes, *wg* expression is absent from the visceral mesoderm. The embryos are between stages 13 and 14 of development; anterior is oriented to the left.

Fig. 6. *wg* protein migrates to the endoderm. (A) In stage 17 embryos *wg* protein (brown stain) is detectable at the apical side of the endoderm (arrowheads), i.e., the side towards the lumen of the midgut, indicating that it has moved from the visceral mesoderm where *wg* protein is seen at an earlier stage (Fig. 3B in van den Heuvel et al. 1989). The adjacent visceral mesoderm cells express *Ubx* (black staining; arrows) The *wg* expressing midgut loop is visible to the posterior of the *Ubx* domain, to the left in the photograph, because the loop has inverted its position during the convolution of the midgut. (B) High magnification view of the central region of the midgut of a stage 15 embryo. *Ubx* protein in the visceral mesoderm nuclei is stained in black (arrowheads); *wg* protein is visualized in brown (arrows). The more posteriorly located *Ubx* protein-containing cells (to the right) also display *wg* protein on their surfaces. (C) Central region of the visceral mesoderm of a stage 13 embryo which has been stained for both *dpp* and *wg* protein (in brown) and *Ubx* protein (in black).
HSU embryos after a heat shock treatment the lab protein is restricted to the outer endodermal layer, where the cells are in direct contact with cells that are producing dpp protein (Fig. 4E,F). The anterior constriction of the midgut and the constrictions separating the gastric caeca are not formed in these embryos, confirming that the appropriate spatial distributions of homeotic and dpp gene products are required for midgut morphogenesis.

In the part of the midgut endoderm posterior to the developing gastric caeca, ectopic dpp protein is sufficient to induce lab expression in the neighboring germ layer (Fig. 4D). However, the ectopic domain of lab protein does not extend as far to the anterior as the ectopic dpp in the heat-shocked HSU embryos (Fig. 4A,C). lab is not expressed in the endoderm of the budding caeca or anterior to them.

Abdominal-A expression is required for the expression of wingless in the visceral mesoderm

In regions of the midgut where cells do not make dpp protein, communication between germ layers is most likely to be mediated by other growth factors. The only other Drosophila growth factor known to be produced in the midgut is the protein encoded by the wg gene. The segment polarity gene wg, the Drosophila homolog of the int-1 oncogene, was found to be expressed in PS 8 of the visceral mesoderm (van den Heuvel et al. 1989). abd-A is required for wg expression in the visceral mesoderm. Embryos homozygous for a null mutation of abd-A (Fig. 5C), or lacking part of (not shown) or the entire bithorax complex (Fig. 5D), do not accumulate wg protein in the visceral mesoderm. Thus wg looks like a good candidate for a target gene positively regulated by abd-A. However, abd-A cannot be sufficient to control the spatial distribution of wg because wg is only expressed in the most anterior part of the broad visceral mesoderm region where abd-A protein is made (Fig. 5A). This function is probably not Ubx as Ubx(E18-28) mutants retain wild-type wg expression (Fig. 5B). However, at least one other BX-C function is required for wg expression because Df(3R)bxd100 embryos lack wg products in PS 8 (Fig. 5E). In contrast to another report (Immerglück et al. 1990), we find that the wg domain overlaps those of Ubx and dpp (Fig. 6A, B, C).

Some visceral mesoderm cells at the anterior rim of the wg domain have Ubx protein in their nuclei and wg protein at the cell periphery (Fig. 6B). This could be due to movement of the wg protein rather than to transcription of both genes in some of the same cells. We have observed movement of wg protein from the visceral mesoderm to the apical side of the endoderm in older embryos (Fig. 6C).

Ubx may not be required for spatially restricted expression of wg in the visceral mesoderm despite the (limited) physical overlap of Ubx and wg products. Embryos homozygous for the Ubx(E18-28) allele, which has 32 base pair deletion at codon 27 (Beachy et al. 1985; Weinzierl et al. 1987) and therefore probably has little or no Ubx function, have wg protein in an unchanged pattern (Fig. 5B) as do HSU embryos in which ectopic Ubx expression has been induced (not shown). However, a function within the bithorax complex other than abd-A is required for normal wg expression. In embryos homozygous for Df(3R)bxd100, which removes the Ubx and the bxd transcription units from the third chromosome, or in embryos bearing Df(3R)bxd100 in trans to Df(3R)P9, which removes all of the BX-C, not only are Ubx and dpp expression gone from the visceral mesoderm, but wg expression is also severely reduced (Fig. 5E).

Discussion

The requirement for homeotic gene function in establishing a wild-type body plan is well documented (reviewed in Mahaffey and Kaufman, 1988); however, the mechanism by which these genes act is just beginning to be elucidated. Part of this mechanism clearly involves inter-regulation among the homeotic genes (Hafen et al. 1984; Struhl and White, 1985; Carroll et al. 1986; Riley et al. 1987; Casanova and White, 1987). Another part of this mechanism is likely to involve the regulation of target genes which implement the program set up by the homeotic genes. These target genes could mediate intercellular interactions as well as intracellular differentiation events. Recently, two putative targets that are not homeotic genes, dpp and wg, have been postulated based on their altered expression patterns in the visceral mesoderm of embryos mutant for Ubx or abd-A (Immerglück et al. 1990; Panganiban et al. 1990b). In this report, we examine the effect of ectopic expression of Ubx during gastrulation on the expression of dpp. We conclude that Ubx and abd-A are sufficient to delimit the spatial expression of dpp in the middle part of the visceral mesoderm. Although we have not proven that dpp is directly regulated by the Ubx protein, this possibility is the simplest explanation for our observations. Further evidence consistent with a direct interaction model comes from experiments in which binding sites for the Ubx protein were found in or near the dpp gene (P.A. Beachy; J. Botas and D.S. Hogness, personal communications).

Ubx is required for dpp expression

In embryos bearing the putative Ubx protein null mutation, Ubx(E18-28), the expression of dpp is reduced in the visceral mesoderm of PS 6 and 7 to a faint band in posterior PS 7. In embryos homozygous for deficiencies of the bithorax complex that remove the Ubx transcript, e.g. Df(3R)bxd100, dpp expression is completely eliminated in the visceral mesoderm of PS 6 and 7. These data indicate that Ubx is required for dpp expression in the central region of the midgut, and are essentially in agreement with the report of Immerglück et al. (1990). They reported that in embryos bearing a Ubx1 mutation in trans to a triple mutation of the bithorax complex that eliminates all its known protein-coding functions (Casanova et al. 1987) there is no dpp expression in the middle of the midgut visceral mesoderm. We are not yet
sure why in embryos homozygous for Ubx<sup>h<sup>28</sup></sup> there is some spatially limited dpp expression, while in the other cases mentioned dpp expression in the midgut has vanished completely. Ubx<sup>h<sup>28</sup></sup> has a deletion of 32 bp which creates a stop codon after only 27 codons and therefore is likely to be a null mutation in Ubx (Weinzierl et al. 1987), but perhaps it has a residual function below detectable levels. Df(3R)bx<sup>119</sup> removes functions in addition to Ubx such as abx, bx, pbx, or bxd; so perhaps one or more of these can affect dpp expression and is also defective in the Ubx<sup>h<sup>28</sup></sup>/triplet mutant. Another mystery is why dpp expression in the posterior part of PS 6, where no Ubx protein is detected, is dependent upon Ubx function. One possibility is that dpp expression in posterior PS 6 is due to limited paracrine autoactivation by the dpp protein coming from PS 7. Such a localized effect would be prevented in more posterior regions by the negative influence of abd-A on dpp.

Ubx is sufficient for dpp expression

When the synthesis of Antp or Ubx products is induced throughout the embryo using a heat shock promotor, the effects of the proteins are limited to specific tissues and to specific regions of the embryo (Gibson and Gehring, 1988; Kuziora and McGinnis, 1988; Mann and Hogness, 1990; Gonzalez-Reyes et al. 1990). For example, only the cuticle of an embryo anterior to the normal region where Ubx is expressed is affected by expression of Ubx in all cells. Ectopic Ubx protein in the posterior abdomen has no detectable effect on cuticle patterning. Thus cells in certain positions are permissive for the actions of the regulator while others are not. The restriction of the effect is at least in part due to competition between homoeotic proteins. The presence of abd-A products prevents Ubx protein from having an effect in the posterior abdomen (Gonzalez-Reyes et al. 1990; Mann and Hogness, 1990). Thus it is not simply the negative regulation of Ubx by abd-A (Struhl and White, 1985) that prevents Ubx from deciving the fate of posterior abdominal cells. Rather, groups of cells that contain both abd-A and Ubx protein form structures characteristic of abd-A function, apparently ignoring the presence of the Ubx protein.

A transposition containing the Ubx cDNA under the control of the heat shock promotor was used to ectopically express Ubx throughout the embryo and the pattern of dpp expression was assayed. Within 3 h after the heat shock, dpp expression in the HSU embryos extends along the entire anterior end of the midgut in the visceral mesoderm. The rapid time course of dpp induction is consistent with a direct interaction. We observe ectopic dpp and ectopic labial expression in stage 15 and stage 16 embryos. At the time of heat shock, these embryos would have been older than 9 h, a stage which is refractory to HSU induced segmental transformations (Gonzalez-Reyes et al. 1990). Thus the response of dpp to Ubx probably occurs in the absence of general changes in segment identity.

The cellular context affects whether a homeodomain protein is capable of activating a particular target gene. It is striking that Ubx does not govern dpp expression in embryonic tissues outside of the anterior half of the midgut visceral mesoderm. The restriction of the activating effect of Ubx could be due to a transcriptional cofactor present only in the visceral mesoderm cells that is required for the Ubx protein to turn on dpp. This cofactor could be another DNA-binding protein, a protein that affects the alternative RNA splicing events that produce different Ubx proteins, a protein that modifies the Ubx protein, or an adaptor protein that interfaces between Ubx and the basic transcription machinery. Alternatively, there could be a block to Ubx activation of dpp in all tissues except the visceral mesoderm. The block could take the form of a repressor protein that competes with Ubx for binding to the dpp gene, a repressor that competes with Ubx protein for contacting the basic transcription apparatus, an enzyme that modifies the dpp gene or the Ubx protein, or a chromatin component that keeps the dpp gene in an inactive or inaccessible state.

abd-A represses dpp via a Ubx-independent mechanism

We have previously shown that in the absence of abd-A function dpp is derepressed in visceral mesoderm of the posterior midgut (Panganiban et al. 1990b). In this report, we demonstrate that the repression of dpp by abd-A is independent of Ubx. In the heat-shocked HSU embryos, dpp is ectopically expressed only in the anterior midgut and not in the visceral mesoderm of the posterior midgut, where abd-A is expressed. This indicates that the repression of dpp by abd-A can take place even in cells that are also expressing Ubx. That abd-A can repress dpp even in the presence of large amounts of Ubx protein indicates that the mechanism by which abd-A negatively regulates dpp is not by negatively regulating Ubx. In heat-shocked HSU-109 embryos, which are deficient for Ubx and abd-A in addition to harboring the HSU transposon, ectopic dpp expression extends over the posterior as well as the anterior midgut, confirming that abd-A represses Ubx induction of dpp in the posterior half of the midgut. The domination of abd-A over Ubx in the control of dpp provides a specific example of target gene regulation that parallels the observations on the effects of ectopic Ubx on cuticle pattern discussed above. It will be interesting to determine whether Ubx protein does in fact bind to dpp DNA in vivo and whether the presence of abd-A interferes with this interaction.

A further indication of the negative regulation of dpp by abd-A is the appearance of a striped pattern of dpp in Df(3R)Ubx<sup>119</sup> embryos which are deficient for Ubx and abd-A. This pattern is reminiscent of segment polarity gene expression patterns and suggests that, in the absence of normal negative regulation of dpp, the gene exhibits an aberrant response to one or more segment polarity genes. In the lateral ectoderm, dpp exhibits a segmentally repeated pattern; thus the gene does possess mechanisms for responding to this sort of pattern information. Presumably, this response is normally overridden in the visceral mesoderm by the
action of the homeotic regulatory proteins. In addition, weak dpp expression is normally present in the visceral mesoderm of PS 9 (Jackson and Hoffmann, in preparation). The PS 9 expression of dpp in the visceral mesoderm is regulated by a completely different set of regulatory elements than those required for dpp expression in PS 7 and the gastric caeca (Jackson and Hoffmann, in preparation). It is not known what regulates dpp expression in PS 9, but in the absence of regulatory input from the homeotics, these unknown factors may be able to generate the striped pattern of dpp expression observed in the Df(3R)Ub \textsuperscript{+} mutants.

That \textit{abd-A} can have positive as well as negative effects on target genes is indicated by the observation that embryos null for \textit{abd-A}, or for the entire BX-C, do not express \textit{wg} in PS 8 of the visceral mesoderm.

However, at least one other function within the BX-C is also required for dpp expression: Embryos that are homozygous for Df(3R)bx \textsuperscript{+} or trans-heterozygous for Df(3R)bx \textsuperscript{a/ab} and therefore still have \textit{abd-A} function, also lack \textit{wg} expression. This function may not be \textit{Ub} because, in \textit{Ub} \textsuperscript{a/ab} mutant embryos, \textit{wg} expression in PS 8 of the visceral mesoderm is normal. However, as is discussed above, \textit{Ub} \textsuperscript{a/ab} may not be null for all \textit{Ub} functions. The absence of ectopic \textit{wg} products in regions of HSU embryos where \textit{abd-A}, \textit{Ub}, and \textit{dpp} are all expressed is consistent with the hypothesis that \textit{Ub} is not the BX-C function required for \textit{wg} expression. Immergötz et al. (1990) have reported that dpp expression in PS 7 is required for \textit{wg} expression in PS 8. Perhaps there is sufficient movement of dpp protein, at levels undetected in the present studies, from PS 7 into the visceral mesoderm of PS 8, to induce \textit{wg} expression in cells expressing \textit{abd-A} and not expressing \textit{Ub}. The overlap we have observed in the \textit{Ub} and \textit{wg} protein domains might be due to an anterior movement of the \textit{wg} protein from its site of synthesis into the most posterior part of PS 7. Experiments using heat shock promoter driven ectopic expression of dpp could be used to test this hypothesis.

\textbf{Reciprocal regulation of dpp and Ub}

Although \textit{Ub} is responsible for localized expression of dpp in posterior PS 6 and PS 7 of the visceral mesoderm, we have also observed an effect of dpp on \textit{Ub} in the visceral mesoderm (Panganiban et al. 1990). We have not established whether \textit{Ub} protein or dpp protein is present first in the visceral mesoderm of PS 7. dpp RNA is clearly detected in the visceral mesoderm by stage 11 as germ band shortening begins (Jackson and Hoffmann, in preparation). We think it most likely that \textit{Ub} initiates dpp expression, and that dpp is required to maintain \textit{Ub} expression in PS 7. Secreted factors, like dpp, with a limited capacity for moving from their site of synthesis, may provide an ideal means of mediating intercellular interactions in a specific region of the embryo. The intercellular signals
may be necessary to maintain particular differentiated cell states among the population of cells, possibly by causing maintenance of homeotic gene expression.

Requirements for lab expression in the midgut endoderm

Expression of dpp in the midgut visceral mesoderm is required for the expression of lab in the underlying endoderm (Immerglück et al. 1990; Panganiban et al. 1990b). We have extended this analysis and find that in order for endoderm cells to express lab, they must have been in contact with the visceral mesoderm cells which express dpp relatively early (i.e. stage 13) during midgut development. The apparent temporal requirement for early exposure to dpp for lab expression may reflect a subsequent commitment by the endoderm cells to specific patterns of differentiation, for which lab expression is a marker.

Early exposure of the endoderm cells to dpp is required, but clearly not sufficient to induce lab. In heat-shocked HSU embryos, ectopic expression of dpp extends over the entire anterior midgut, while lab expression is restricted to the endoderm of the midgut between the gastric caeca and the second constriction. The distribution of other factors (e.g. dpp transport protein or receptor, signal transduction machinery, and/or specific transcription factors) may determine whether particular endoderm cells can respond to dpp. Differences in the responses of parts of the midgut endoderm to dpp were noted previously (Panganiban et al. 1990b). dpp is expressed in wild-type embryos in the visceral mesoderm of the gastric caeca as well as the visceral mesoderm of PS 6 and 7, yet lab expression is only detected in the endoderm cells of PS 6 and 7. The dpp protein has been shown to migrate from the visceral mesoderm across the endodermal cell layer in PS 6 and 7, however, no migration of dpp protein has been observed at the caeca (Panganiban et al. 1990; Panganiban and Hoffmann, unpublished observations). It may be that the dpp protein simply does not migrate to the endoderm at the anterior end of the midgut, and that if it did, lab would be induced in the underlying endoderm.

Gene interactions in the midgut

The interactions among the genes discussed above and by Panganiban et al. (1990b) are summarized in Fig. 8. Immerglück et al. (1990) have independently arrived at many of the same conclusions. At the anterior end of the midgut, both dpp and Scr expression are required for the gastric caeca to form. dpp expression prevents Scr expression in the cells that form the gastric caeca. As the cells expressing Scr act at a distance on the cells that form the gastric caeca, we expect that an as yet unidentified secreted factor, perhaps another growth factor homolog, may be made in response to Scr. Antp expression positively regulates Scr expression, also at a distance, and Antp expression is required for the
anterior midgut constriction to form. Mutations in Scr or Antp do not affect the expression of the other homeotic genes, or of dpp or wg. In the adjacent region, we propose that Ubx causes dpp expression, which positively maintains Ubx expression in the visceral mesoderm cells and induces labial expression in the adjacent endodermal cells. Both dpp and Ubx expression are required for these cells to form the middle constriction. In the posterior midgut, abd-A expression negatively regulates Ubx and dpp, and positively regulates wg. The domain of wg expression is restricted to PS 8 — only a portion of the visceral mesoderm cells expressing abd-A — by as yet unidentified factors. It has been reported that movement of the wg protein to the apoposing region is involved in maintaining labial expression in the endoderm (Immergötz et al. 1990). The molecular basis of these interactions remains to be determined. These studies present an initial picture of the regulatory circuitry in the midgut of the embryo and establish growth factor homologs in Drosophila as target genes for the pattern information inherent in localized homeotic gene expression. It is likely that the homeotic gene products themselves also regulate the expression of other molecules including cytoskeletal components, specialized enzymes, and cell surface components. Our understanding of how specific morphological events occur in response to the homeotic genes will require continued progress in identifying the target genes of the homeotic themselves and in identifying the genes regulated indirectly by molecules like dpp and wg.

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