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Regional repression of a *Drosophila* POU box gene in the endoderm involves inductive interactions between germ layers

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SUMMARY

An induction process occurring between the mesodermal and the endodermal germ layers has recently been described in the regulation of the *Drosophila* homeotic gene *labial (lab)*. We report here that proper spatial regulation of the *Drosophila* POU box gene *pdm-1* products also involves interaction between these two germ layers. *pdm-1* transcripts are initially present in both the anterior and the posterior endodermal midgut primordia. Upon fusion of the two primordia, transcripts disappear from two regions in the endoderm, a central domain and an anterior domain. The anterior repression domain of *pdm-1* is independent of the expression of known homeotic genes and genes encoding secreted signalling molecules in the visceral mesoderm, both for its positioning and its repression. Repression in the central domain requires both the homeotic gene *Ultrabithorax* (*Ubx*) and the *decapentaplegic* (*dpp)* gene, which encodes a secreted protein. Both of these genes are also required for *lab* induction. However, the analysis of *pdm-1* expression in various mutant backgrounds indicates that the regulation of *lab* and *pdm-1* across germ layers is controlled by different genetic cascades. Our study indicates that *dpp* is not the signal that dictates central *pdm-1* repression across germ layers and suggests that in the same midgut region, different signalling pathways result in the differential activation or repression of potential transcription factors.

Key words: *Drosophila* embryogenesis, endoderm, homeotic genes, POU-box

INTRODUCTION

During the early development of a *Drosophila* embryo, a hierarchical genetic network plays a key role in determining positions along the anteroposterior axis (for a review, see Pankratz and Jäckle, 1990; St. Johnston and Nüsslein-Vollhard, 1992; Ingham and Martinez-Arias, 1992). Maternally deposited positional cues are interpreted and refined by a set of segmentation genes and their gene products. The establishment of segments is followed by the assignment of specific functions to individual segments through the differential activation of homeotic genes along the anteroposterior axis (see McGinnis and Krumlauf, 1992).

Although homeotic gene products are crucial for the establishment of segment-specific identities in ectodermal structures, it has been shown only more recently that homeotic gene products are also activated region specifically in the mesodermal cell layer, suggesting that they play a similar role in this germ layer. In the mesoderm, and particularly well described in the visceral mesoderm (VM), homeotic genes are regulated by a set of regulatory processes that are distinct from the ones that rule in the ectoderm (Bienz and Tremml, 1988; Tremml and Bienz, 1989a; Reuter and Scott, 1990).

In contrast to the primordia of the ectoderm and the mesoderm that extend along most of the anteroposterior axis, the endoderm develops from two primordia at the anterior and posterior embryonic poles outside the functional domains of most segmentation genes. During later developmental stages, the two primordia invaginate, start to grow towards each other and eventually meet to form a continuous tube. Before the two primordia fuse, VM cells laterally join the extending endoderm. Together, the endodermal gut epithelium and the surrounding VM form the embryonic midgut.

Although region-specific expression of several regulatory genes can be observed in the growing primordia of the developing endoderm (Mlodzik et al., 1985, 1988; Diederich et al., 1989), more precise positional information along the anteroposterior axis seems to be provided to the endoderm via an induction process across germ layers involving the adjacent VM (Immerglück et al., 1990; Reuter et al., 1990). This view is based on the finding that the expression of a functional *Ubx* gene product in the VM is required and sufficient to induce high levels of lab protein in the underlying endoderm (Immerglück et al., 1990; Reuter et al., 1990). In the VM itself, *Ubx* controls the expression of *dpp*, a member of the transforming growth factor β *(TGF-β)* family, which is required for and thought to mediate the induction process (Immerglück et al., 1990; Reuter et al., 1990). Consistent with this hypothesis, the *dpp* gene product can be found to migrate from the VM to

the adjacent endodermal cells that express high levels of *lab* (Panganiban et al., 1990).

At present, only the homeotic gene *lab* has been shown to respond to induction in the *Drosophila* midgut. We report here that the endodermal expression pattern of the *Drosophila* POU box gene *pdm-1* is also modulated by interactions between the two germ layers. *pdm-1* transcripts are initially present in both primordia of the endoderm, but transcript levels drop dramatically in two regions, an anterior and a central domain, upon fusion of the two primordia. *pdm-1* repression in the anterior domain is independent of the expression of known homeotic genes and genes encoding secreted molecules in the VM. Repression of the *pdm-1* gene in the central domain requires both *Ubx* and *dpp*, as does *lab* for induction. However, more detailed studies revealed that *lab* and *pdm-1* regulation across germ layers are controlled by different genetic cascades and suggest that *dpp* might not mediate the repressive effect on *pdm-1*. This is best illustrated by the finding in *odd-paired (opa)* mutants that central repression of *pdm-1* does not occur despite the presence of *dpp* activity in the VM and *lab* induction in the endoderm.

MATERIALS AND METHODS

General methods

Preparation of genomic DNA, isolation of DNA from lambda phages and plasmids, restriction endonuclease digestions, gel electrophoresis and blotting of DNA onto nitrocellulose membranes were performed as described by Maniatis et al. (1982). Several POU-box-containing phages were isolated from a genomic *Drosophila* Canton S library in Charon 4A (Maniatis et al., 1978). The cDNA clones were isolated from a 3-12 hour embryonic cDNA library (kindly provided by L. Kauvar). All genomic and cDNA fragments isolated from phages were subcloned into Bluescript vectors (Stratagene) or m13 vectors. DNA was sequenced by the procedure of Sanger et al. (1977).

Our cDNA sequences are virtually identical to those reported by Billin et al. (1991). Our sequence starts at nucleotide 139 of their *pdm-1* cDNA clone (clone c33i-c6) and ends at nucleotide 2774. Only one single and one double nucleotide substitutions as well as a single one nucleotide gap are found in the 3′ untranslated region.

In situ hybridization

In situ hybridization to whole-mount embryos was performed as described by Tautz and Pfeifle (1989) with the following modifications. In the labelling reaction, a random primer concentration of 5 mg/ml was used and the reaction was incubated overnight at 14°C, then 2 U of Klenow fragment were added and the reaction continued for 4 hours at room temperature. The anti-digoxigenin antibody was preabsorbed against a large volume of fixed embryos in a 1:10 dilution overnight at 4°C. The following probes were used for hybridizations. For *pdm-1*, a 1.7 kb *Eco*RI fragment encompassing the entire coding sequence was labelled. Under our conditions, no crosshybridization to an additional POU box gene located at 33F was observed. For *wg*, we have used a 2.9 kb cDNA insert (Rijsewijk et al., 1987). For *lab*, the entire insert of the cDNA clone c241 was used (Mlodzik et al., 1988). For *dpp*, a cDNA clone containing an approximately 4.5 kb insert covering the entire coding sequence was labelled (St. Johnston et al., 1990). The *lacZ* probe consisted of a 2 kb fragment. Double antibody/in situ hybridizations were done essentially as described by Manoukian and Krause, (1992) with antibody staining (using a monoclonal β-gal antibody from Promega) done before the in situ hybridization with *pdm-1*. The biotinylated secondary antibody was detected with the Vectastain kit (Vector Laboratories). Embryos used for the double antibody/in situ hybridization contained two wild-type copies of the *lab* gene as well as a P-element containing 6.3 kb of *lab* upstream sequences fused to a bacterial β-gal gene (see Tremml and Bienz, 1992). Expression of this fusion gene is virtually identical to *lab* expression in stage 14 embryos (Tremml and Bienz, 1992, and data not shown). Using a lab antiserum kindly provided by Tom Kaufman, we have also observed this overlap in wild-type late stage 13 embryos (data not shown). Younger embryos did not accumulate enough detectable *lab* protein to identify definitively a potential overlap between *lab* protein and *pdm-1* transcript.

Identification of mutant embryos

Unambiguous identification of homozygous mutant embryos was essential, especially for mutants in which no dramatic changes in the *pdm-1*-expression pattern was observed. In the case of *Scr*, *Antp*, *abd*-A and Pc^3 mutant stocks, we have used a TM2 balancer chromosome carrying a p(lArB) enhancer detector that is strongly expressed throughout the embryo in stages 11 to 13. This chromosome was isolated in a screen described by Bellen et al. (1989). Embryos were hybridized simultaneously with a *lacZ* and a *pdm-1* probe. For *lab*, we have made use of a mutant stock that is homozygous for a *lab*-β-gal fusion construct on the second chromosome (kindly provided by Gaby Tremml and Mariann Bienz). This construct contains 6.3 kb of *lab* upstream sequences driving β-gal expression in the endoderm (Tremml and Bienz, 1992). As the midgut expression of this construct is entirely *lab*-dependent, β-gal activity is only present in lab^+ embryos allowing easy identification of lab^- embryos. For definitive identification of dpp^{s4} and $wg^{IL114ts}$ homozygous mutant embryos, the chromosomes containing these mutations were balanced over a CyO chromosome that contained a hindgut/anal pad-specific β-gal fusion construct (U.W., unpublished data). For the $wg^{IL114ts}$ experiment, embryos were grown at the permissive temperature (18°C) for between 4 hours and 14 hours and then at the nonpermissive temperature (29°C) for 5 hours.

Fly strains

The following strains were used for analysis: $Antp^{rw10}$ (Wakimoto and Kaufman, 1981); Scr^4 (Lewis et al., 1980a,b); *Ubx*1 (Bender et al., 1983); triple chromosome Ubx^{mx12} *abd*-A^{m1} *Abd*-B^{m8} (Casanova et al., 1987); *abd*-A^{m1} *Abd*-B^{m8} (Casanova et al., 1987); Pc^3, DfP9, Df109 (Lewis, 1978); wg^{cx4} (Baker, 1987); lab^{vd1} (Diederich et al., 1989); dpp^{s4} (St. Johnson et al., 1990); opa^{IIP32} (Tearle and Nüsslein-Volhard, 1987); eve^{I034} (Nüsslein-Volhard et al., 1984); $wg^{IL114ts}$ (Baker, 1988).

RESULTS

Isolation of a *Drosophila* POU box gene

In order to identify putative *Drosophila* POU box genes, we screened a *Drosophila* genomic library with a DNA fragment encompassing the POU domain of a human *Oct*-2 cDNA clone (Müller-Immerglück et al., 1988; see Materials and Methods). As judged by restriction mapping of individual phage inserts, only one class of phages that gave a strong signal under high-stringency conditions was recovered. A POU-box-containing fragment of this isolated genomic DNA was then used to screen a *Drosophila* cDNA

library prepared from 3-12 hour embryonic RNA (Poole et al., 1985). Several clones were obtained that fell into a single class of cDNAs. The two longest overlapping inserts were sequenced. The longest cDNA clone characterized contains a 1803 bp open reading frame that has the potential to code for a 601 amino acid protein. At its extreme C-terminal end, the open reading frame encodes a POU domain in which 92% of the residues of the POU-specific domain and 81% of the residues of the homeodomain are identical to *Oct*-2 (Müller-Immerglück et al., 1988; see also Materials and Methods). The chromosomal location of the gene was mapped to 33F using in situ hybridization to salivary gland polytene chromosomes (data not shown). During the course of our studies, other groups have reported DNA sequences of *Drosophila* POU box genes (Johnson and Hirsh, 1990; Billin et al., 1991; Dick et al., 1991; Lloyd and Sakonju, 1991). Comparing the sequences of our cDNAs with those sequences revealed that we had characterized the transcript of a gene named *pdm-1* (Billin et al., 1991; Lloyd and Sakonjo, 1991) or *dPOU-19* (Dick et al., 1991). Using the nomenclature of Billin et al. (1991), we refer to the gene the regulation of which we investigated in this study as *pdm-1*.

Expression pattern of *pdm-1* at the RNA and the protein level

We have analysed the expression pattern of *pdm-1* using in situ hybridization to whole-mount embryos (Tautz and Pfeifle, 1989). *pdm-1* transcripts are first detected shortly before cellularization (stage 5; Campos-Ortega and Hartenstein, 1985) as a band of expression extending from 20% to 50% egg length (Fig. 1A; 0% egg length is located at the posterior pole). Upon cellularization, this band of expression resolves into two stronger stripes (Fig. 1B). During germ band elongation, each of the two bands splits again. Afterwards, transcripts slowly fade away during stage 8 (Fig. 1C). Weak expression is now detectable in the anterior head region at a location that later gives rise to the clypeolabrum. Before full germ band extension, at stage 9, strong segmentally repeated expression is observed in the region of the neurectoderm. This pattern evolves in a dynamic fashion until at stage 10, virtually all cells of the neurogenic region contain high levels of *pdm-1* transcripts (Fig. 1D; for a more detailed description of the expression pattern during these stages, see Dick et al., 1991). At stage 11, ectodermal staining has disappeared and transcripts are detected in a subset of neuroblasts in the developing CNS. During germ band retraction, *pdm-1* transcripts are detectable in the developing anterior and posterior midgut primordia (Fig. 1E). The staining is quite uniform and extends throughout both primordia until they fuse during stage 12 (Fig. 1F). Shortly after fusion, the uniform staining over the entire midgut endoderm is dramatically modified. Transcripts disappear from two regions, an anterior domain and a more central domain (Fig. 1G). This expression pattern in the endoderm is essentially maintained without much alteration during stages 13 and 14 in which the midgut closes up on both the dorsal and the ventral side (Fig. 1H). However, the expression domain in the region of the proventriculus splits into two strongly labeled bands during these stages (data not shown). During stages 15 and 16, when the three midgut constrictions are forming, the extension of *pdm-1* transcript distribution in the posterior midgut slightly changes its anterior border, which moves somewhat posteriorly (Fig. 1I). Strong expression is still detectable in most of the midgut, with the exception of the anterior repression domain, which lies posterior to the proventriculus as well as the central repression domain around the second midgut constriction (Fig. 1I). Within the third midgut convolution, *pdm-1* transcripts are more abundant in the posterior part than in the anterior part. Transcript levels are highest in the fourth midgut convolution (Fig. 1I). In stages 15 and 16, expression is also apparent in certain cells in the head, the CNS and the PNS (data not shown; see also Dick et al., 1991). The expression pattern of *pdm-1* in the midgut has not been described previously (see Dick et al., 1991; Lloyd and Sakonjo, 1991), possibly because long protease treatment during the preparation of embryos for whole-mount in situ hybridization is required to detect strong staining in the interior of the embryo.

We generated polyclonal antisera against an *E. coli*-produced pdm-1 polypeptide and analysed the protein distribution during the early stages of *Drosophila* development. The expression domains observed at the protein level are virtually identical to the previously described domains of expression of *pdm-1* transcripts. This also holds true for the modulation of the transcript distribution pattern upon midgut fusion; the protein level drops rapidly in the two regions from which the transcripts disappear (data not shown).

For simplicity, we use the term ‘repression’ in the following sections to describe the disappearance of *pdm-1* transcripts upon midgut fusion. However, we do not know in molecular terms whether the phenomenon involves an active repression effect at the transcriptional level, a lack of further transcriptional activation or a localized posttranscriptional increase in degradation of the RNA.

pdm-1 is repressed adjacent to visceral mesoderm cells secreting known extracellular signals

We have mapped the domains of *pdm-1* expression in the endoderm with respect to well-characterized domains of expression of other genes in the endoderm or the adjacent VM. For this purpose, we have used probes specific for *wingless (wg)*, *dpp* and *lab*.

The *wg* gene is activated in the anterior part of parasegment 8 of the VM, a process that requires the homeotic gene *abdominal-A* (*abd*-A) (Immerglück et al., 1990; Reuter et al., 1990; see also Fig. 7). Whole-mount in situ hybridization on wild-type embryos using both a *pdm-1* and a *wg* probe demonstrated that immediatly after repression becomes evident, the anterior border of cells expressing *wg* transcripts in the VM is in close proximity to the anterior border of *pdm-1* expression in the posterior part of the endoderm (Fig. 1J). This indicates that *pdm-1* repression in the central domain occurs in endodermal cells roughly adjacent to VM cells of parasegment 7.

dpp is expressed in two domains of the VM, an anterior domain covering the region from which the gastric caeca

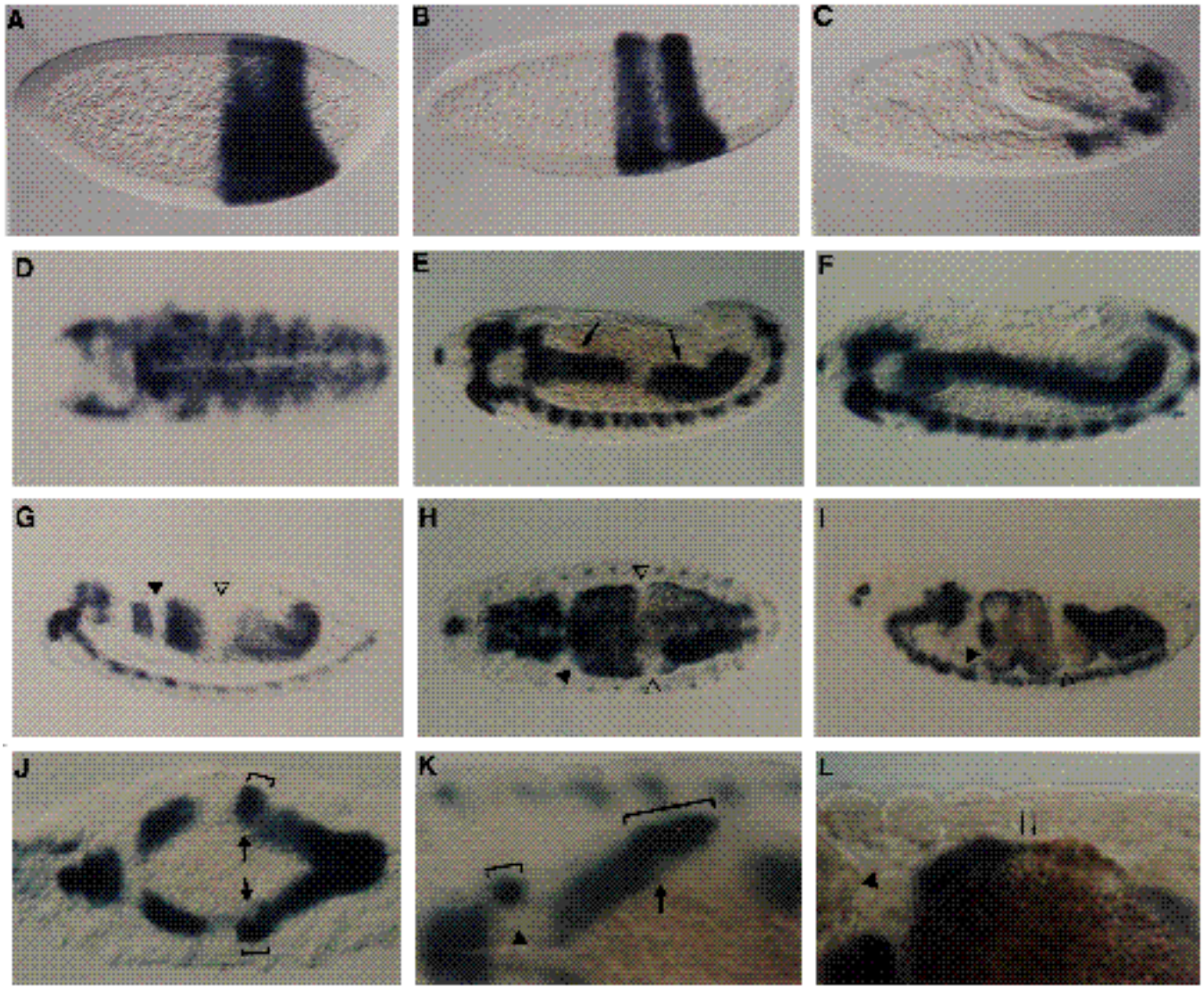

Fig. 1

develop and a central domain mapped to VM parasegment 7 (Immerglück et al., 1990; Reuter et al., 1990). To further map *pdm-1*-expression domains upon midgut fusion, double hybridizations using *dpp* and *pdm-1* probes were performed. *pdm-1* repression in both the anterior and the posterior domain occurred in endodermal cells adjacent to *dpp*-expressing VM cells, but none of the borders observed in the VM coincided precisely with the *pdm-1* borders in the endoderm (see Fig. 1K).

lab is expressed at high levels in endodermal cells adjacent to VM parasegment 7 in stage 13 embryos (Immerglück et al., 1990; Reuter et al., 1990). Double hybridizations using *lab* and *pdm-1* probes were performed and analysed with respect to the extent and intensity of the signal in the endoderm immediately after *pdm-1* repression becomes apparent. The hybridization pattern demonstrated that all cells of the central domain where *pdm-1* expression is shut off contain high levels of *lab* transcripts, i.e. all cells in the middle portion of the midgut contain either *pdm-1* and/or *lab* transcripts (data not shown, see below). To investigate whether certain cells express both *lab* and *pdm-1*, we have performed double antibody/in situ hybridizations. For this purpose, we have made use of a strain carrying a P-element which contains 6.3 kb of 5′ flanking DNA of the *lab* gene fused to a bacterial β-gal gene (Tremml and Bienz, 1992). In this line, β-gal is expressed in the midgut in a pattern virtually indistinguishable from that of the endogenous *lab* gene although slightly lagging behind *lab* induction (see Tremml and Bienz, 1992). Using β-gal antibodies and a *pdm-1* probe (see Materials and Methods), we found that the anteriormost two or three β-gal-expressing midgut cells overlap with *pdm-1* transcript-containing cells; no overlap (as well as no gap) is seen in the posterior part of the expression domain (Fig. 1L). Similar results were obtained using a *lab* antiserum instead of a β-gal antibody (data not shown; see Materials and Methods). In stage 16 embryos, *pdm-1* transcripts (Fig. 1I) and *lab* protein (Immerglück et al., 1990; Reuter et al., 1990) also overlap as both are expressed in parts of the second midgut convolution.

Fig. 1. Expression pattern of *pdm-1* in early embryonic stages and mapping of *pdm-1*-expression domains in the midgut. (A-I) Whole-mount embryos hybridized with a *pdm-1* probe as described in Materials and Methods. (A) Lateral view of a early stage 5 embryo. (B) Lateral view of a late stage 5 embryo. (C) Lateral view of a stage 8 embryo; the early staining pattern slowly disappears. (D) Dorsal view of a stage 10 embryo focused on the surface. Abundant transcripts can be detected in the region of the neurectoderm. (E) Lateral view of a late stage 12 embryo; ectodermal staining is no longer detected at this stage. Expression is now seen in the extending anterior and posterior midgut (arrows). (F) Lateral view of a stage 13 embryo; uniform staining is visible in the fused midgut. (G) Lateral view of a slightly older stage 13 embryo; the previous uniform midgut staining is interrupted in two regions, a more anterior region and in a centrally located domain (closed and open arrowheads, respectively). (H) Dorsal view of a stage 14 embryo. Repression is still evident in the anterior domain and in the central domain. The central repression domain extends from somewhat anterior to the second constriction (which forms first) to slightly posterior to this constriction. Previous uniform staining in the region of the proventriculus has split up into two domains of expression (not shown). (I) Dorsal view of a stage 16 embryo. Staining is still detectable in the proventriculus. Transcripts are detected in most of the midgut, with the exception of the anterior and the central repression domain. Staining is weaker in the third convolution and has become very strong in the fourth convolution. In general, stage 15 and 16 embryos have to be stained much longer to detect *pdm-1* transcripts in the midgut at levels comparable to earlier stages. This might be due to the whole-mount in situ procedure or to a decrease in transcript level.

The RNA-expression domains of *pdm-1* in the endoderm were mapped in stage 13 embryos with respect to the distribution of *wg* (J) and *dpp* (K) transcripts by means of double hybridization with a *pdm-1* probe. Note that *wg* and *dpp* transcripts are present in the VM, whereas *pdm-1* transcripts are localized in the endoderm. The central repression domain is located anterior to the *wg*-expression domain (J; brackets indicate the extent of *wg* transcript distribution in the VM, the arrows indicate the anterior border of the posterior *pdm-1*-expression domain) and adjacent to the *dpp*-expression domain (K; brackets indicate the extent of *dpp* transcript distribution in the VM, the arrow indicates the anterior border of the central repression domain). Although the anterior as well as the posterior borders of the central *pdm-1* repression domain do not perfectly align with those of *dpp* in the adjacent VM, double hybridizations using a *pdm-1* and a *lab* probe indicate that all the cells from which *pdm-1* transcripts disappear are responsive to *dpp* as evidenced by the induction of high levels of *lab* transcripts (data not shown; see L). The anterior *pdm-1* repression domain is also located adjacent to *dpp*-expressing VM cells (arrowhead in K). Using a strain that expresses β-gal under the control of promoter/enhancer sequences of the *lab* gene, we have analysed the distribution of β-gal protein with respect to the *pdm-1*-expression pattern (L, see also Materials and Methods). *pdm-1* transcript and β-gal protein overlap in 2-3 cells in the anterior β-gal-expression domain (arrows in L). No gap and no overlap of expression is seen in the posterior part. Stages were determined according to Campos-Ortega and Hartenstein (1985). In all views, anterior is to the left.

Taken together, the double hybridizations and the double antibody/in situ stainings demonstrate that central *pdm-1* repression occurs in endodermal cells adjacent to VM cells of parasegment 7. However, both the *dpp* and the *lab*-expression domains overlap anteriorly with *pdm-1*-expressing cells, indicating that endodermal *pdm-1* repression occurs only in cells adjacent to the middle and posterior portion of VM parasegment 7. This suggests that *lab* induction and *pdm-1* repression might be uncoupled, at least in the anterior *lab*-expression domain (see below). In the anterior repression domain, *pdm-1* repression also occurs in endodermal cells adjacent to a *dpp*-expression domain in the VM (schematically shown in Fig. 7).

Ubx is required for *pdm-1* repression in the central domain of the midgut

Previous studies on the regulation of expression of the homeotic gene *lab* in the endoderm have demonstrated the occurrence of an induction process between the VM and the endoderm (Immerglück et al., 1990; Reuter et al., 1990; see also Fig. 7). The close coincidence between the parasegmental expression boundaries of transcripts synthesized in the VM and the domains of repression of *pdm-1* in the endoderm (Fig. 1) as well as the time of repression (upon midgut fusion when the VM adheres to and interacts with the endoderm) suggest that the spatial limits of repression might also be imposed by restricted factors synthesized in the VM.

The homeotic genes *Sex combs reduced (Scr), Antennapedia (Antp)*, *Ubx* and *abdominal-A* (*abd*-A) are expressed in adjacent and nonoverlapping parasegmental domains of the VM along the anteroposterior axis (Tremml and Bienz, 1989a; see Fig. 7). Using whole-mount in situ hybridization to embryos of various mutant fly stocks (carrying either a mutation in a single homeotic gene or mutations/deletions of several homeotic genes), we investigated the expression pattern of *pdm-1* in the absence of functional homeotic gene products. In each case, the mutant genotype of the embryos was confirmed either by an altered pattern of expression of known gene products (transcripts) or by the absence of a marker transcript synthesized only from the balancer chromosome of the mutant fly stocks (see legend of Fig. 2 and Materials and Methods). Due to the relatively weak expression of *pdm-1* in stages 15 and 16 (see Fig. 1 and legend), we concentrated on the characterization of the effects of homeotic mutations immediately upon midgut fusion.

Removing functional *Scr* or *Antp* product does not detectably affect the expression pattern of *pdm-1* in the endoderm (Fig. 2B,C). The same result is obtained when both genes are removed simultaneously by a large chromosomal deletion (Df (3R)4SCB; Howard and Ingham, 1986; data not shown). In contrast, removing the entire Bithorax complex (BX-C; including *Ubx*, *abd*-A and *Abdominal-B (Abd*-B)) results in a clear change in endodermal *pdm-1* expression; repression in the central domain no longer occurs in mutant embryos (Fig. 2D). Essentially the same result was obtained when *Ubx* and *abd*-A (Fig. 2E) or only *Ubx* were inactivated (Fig. 2F,G). This demonstrates that a functional *Ubx* gene product is required for *pdm-1* repression in the central domain. Neither *abd*-A (Fig. 2H; see also below) nor *Abd*-B (data not shown) are required for repression.

We conclude from these experiments that *Ubx* expression is essential for the repression of *pdm-1* in the endo-

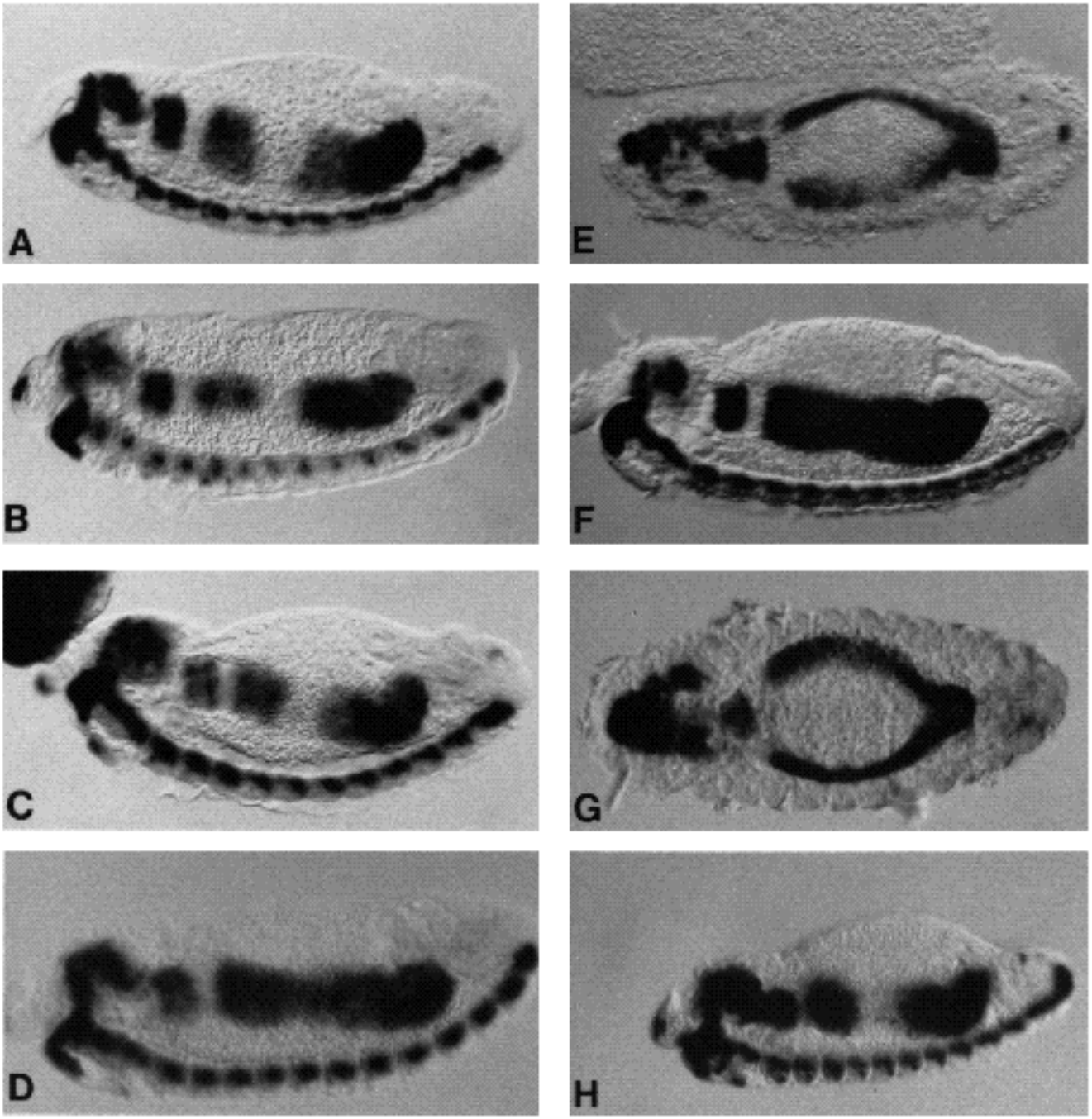

Fig. 2. *pdm-1*-expression in the midgut of homeotic mutants. *pdm-1*-expression in mutant stage 13 embryos was investigated using in situ hybridization to whole-mount embryos. (A) Wild-type embryo. (B) Homozygous *Scr*^{4} and (C) homozygous *Antp*^{rw10} mutant embryos. No change is seen in the expression pattern as compared to wild type. (D) Homozygous DfP9 mutant embryo. Although repression is still observed in the anterior domain, repression no longer occurs in the central domain. The apparently lower transcript level in the central midgut portion results from generally stronger staining in the anterior portion of the midgut (data not shown; see also Fig. 1G). The same result was obtained in homozygous Df109 mutant embryos (E). (F) *Ubx*1/*Ubx*^{mx12} *abd*-A^{m1} *Abd*-B^{m8}. No repression in the central domain. The same result was obtained when *Ubx*1 was analysed over a Df109 (G) or a DfP9 chromosome (data not shown). Homozygous *Ubx*1 embryos show a slightly weaker derepression in the midgut (data not shown). (H) homozygous *abd*-A^{m1} embryo. No change in the repression pattern. *abd*-A^{m1}/Abd-B^{m8} double mutants and *Abd*-B^{m8} alone did not show any change in the expression pattern of *pdm-1* in the midgut (data not shown). Mutant embryos were identified as described in Materials and Methods.

derm of stage 13 embryos and that none of the other homeotic genes studied here is involved in the modulation of the *pdm-1*-expression pattern shortly after midgut fusion. In addition, none of these homeotic genes was shown to be required for the activity of the *pdm-1* gene outside the repressed domains in stage 13 embryos.

dpp is required for full repression in the central domain of the midgut

The above experiments have demonstrated that *pdm-1* repression in the central domain is dependent on *Ubx* function. *Ubx* is known to be required for the expression of *dpp* in the VM, which in turn seems to mediate the induction of *lab* in the endoderm (Immerglück et al., 1990; Reuter et al., 1990; Panganiban et al., 1990). As shown in Fig. 1K, *dpp* is expressed in the VM adjacent to both regions of the endoderm in which *pdm-1* is repressed and could therefore mediate this negative effect.

In previous studies (Immerglück et al., 1990; Panganiban et al. 1990), it has been shown that dpp^{s4} mutant embryos lack *dpp* expression in the VM whereas earlier *dpp* functions do not seem to be affected. Using dpp^{s4}/CyO flies with a hindgut/anal pad-specific β-gal marker on the balancer chromosome (which enables us to distinguish dpp^{+} from dpp^{-} embryos, see Materials and Methods and Fig. 3A), the requirement for *dpp* for *pdm-1* repression was investigated. We found that *dpp* is required for full repression in the central domain but dispensable in the anteriorly located domain (Fig. 3B,C). As *dpp* transcripts are clearly absent in the VM of dpp^{s4} embryos (Panganiban et al., 1990; our unpublished observation), we conclude that while *dpp* is required for full repression to occur in cells adjacent to VM cells of parasegment 7, it is not required for repression in the anterior domain. However, careful inspection of a large number of dpp^{s4} mutant embryos revealed that repression is not always completely abolished (see Fig. 3C and data not shown). Comparing *pdm-1* repression in dpp^{s4} and *Ubx* mutant embryos, it seems likely that the complete lack of repression observed in *Ubx* mutant embryos (see Fig. 2F,G) might not be solely due to the absence of *dpp* in this mutant background. Other factors required for repression might also be affected in *Ubx* mutants and contribute to the observed strong effect on the central repression domain (see Discussion).

A second secreted protein, which is expressed in the center of the midgut VM, is encoded by the gene *wg* (Immerglück et al., 1990; Reuter et al., 1990). *wg*, as described above, is expressed adjacent to and posterior of *dpp* in the VM. Whole-mount in situ hybridizations on wg^{ts} mutant embryos using a *pdm-1* probe revealed that repression in the central domain still occurs and therefore does not strictly require *wg* (Fig. 3D; see also Figure legend and Materials and Methods). This result is consistent with that observed in *abdA* mutant embryos, which also retain central *pdm-1* repression (Fig. 2H) despite the absence of *wg* expression in the VM (Immerglück et al., 1990; Reuter et al., 1990).

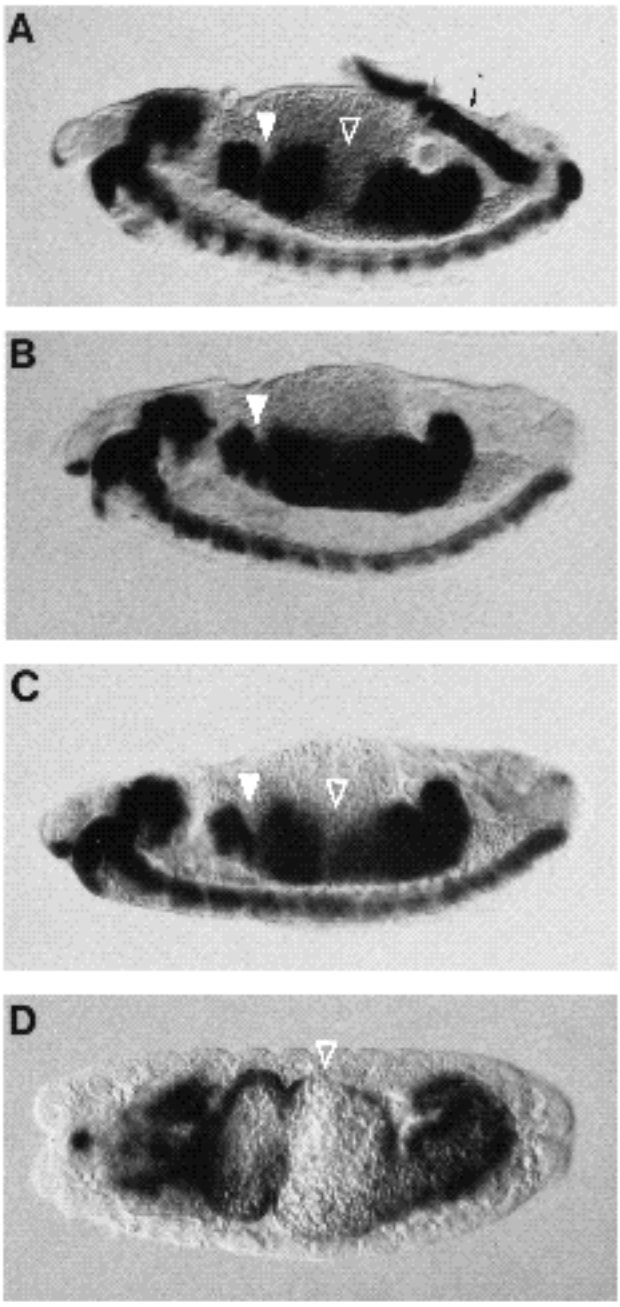

Fig. 3. *pdm-1* expression in embryos mutant for putative secreted signalling molecules. *pdm-1* expression in stage 13 embryos lacking known extracellular signals in the VM was investigated using hybridization to whole-mount embryos. (B,C) Embryos homozygous for the allele s4 lack *dpp* expression in the VM. *pdm-1* repression is strongly diminished, although not completely abolished in the central domain (compare the embryo shown in B with the embryo shown in C; A shows an embryo of the same experiment which contains at least one functional copy of the *dpp* gene, as evidenced by the hindgut/anal pad staining (arrow)). The absence of *dpp* does not perturb repression in the anterior domain (closed arrowheads in A, B and C). (D) Homozygous $wg^{IL114ts}$ embryo grown at the permissive temperature (18°C) for approximately 14 hours after egg laying and 5 hours at the nonpermissive temperature (29°C; see Materials and Methods). Central repression is not affected by the absence of *wg* in the VM. Note the almost normal segmentation of the embryo (as reflected by the number of the segmental grooves formed) and the lack of the formation of the second midgut constriction. This is consistent with the expression of a functional *wg* gene product during the early segmentation process and the inactivation of the wg protein during the process of midgut fusion. The anterior midgut does not properly form in $wg^{IL114ts}$ mutant embryos subjected to our temperature-shift protocol and the effect of *wg* on *pdm-1* repression could therefore not be investigated. Homozygous mutant embryos were identified as described in Materials and Methods.

Fig. 4. *pdm-1* expression in *lab* mutant embryos. *pdm-1* expression is not changed in stage 13 lab^{vd1} embryos as compared to wild type. *lab* homozygous mutant embryos were identified as described in Materials and Methods.

lab is not required for repression in the central domain of the midgut

Ubx and *dpp* are both required for the induction of the homeotic gene *lab* in endodermal cells adjacent to parasegment 7 VM cells. Thus, *lab* is a candidate for a putative transcription factor that might mediate the *Ubx/dpp*-dependent repression of *pdm-1* in the central portion of the midgut endoderm. However, we found that *pdm-1* repression is not altered in embryos homozygous for a *lab* null mutation (Fig. 4).

lab induction and *pdm-1* repression do not use the same genetic cascade

It has previously been shown that the *abd*-A gene is required to set the posterior limit of *Ubx* expression in the VM (Bienz and Tremml, 1988; Immerglück et al., 1990; Reuter et al., 1990). In the absence of *abd*-A, *Ubx* extends to the posterior end of the midgut (Bienz and Tremml, 1988). This results in an expansion of the *dpp*-expression domain towards the posterior end of the midgut (Fig. 5B) and a concomitant extension of the endodermal *lab*-expression domain (Fig. 5C) (Immerglück et al., 1990; Panganiban et al., 1990). In contrast to *lab*, no change is seen in the *pdm-1* pattern in stage 13 embryos, as for example a repression in more posterior endodermal cells (Figs 2H, 5D). *Ubx* can also be ectopically activated in the VM anterior to parasegment 7 in a *Pc* mutant background (Immerglück et al., 1990). Ectopic activation of *Ubx* in the VM anterior to parasegment 7 again results in ectopic expression of *dpp* in the VM and the induction of the *lab* gene in the anterior endoderm (Immerglück et al., 1990; data not shown). As shown in Fig. 5E, *pdm-1* expression is unchanged in *Pc*3 mutants, and no repression is observed in the region between the anterior and the central repression domain. We conclude from these experiments that, in contrast to *lab* induction, ectopic *Ubx* and *dpp* expression in these mutant backgrounds do not result in repression of *pdm-1* in endodermal cells anterior or posterior to VM parasegment 7 and suggest that they might also not be sufficient for repression in the central domain itself (see

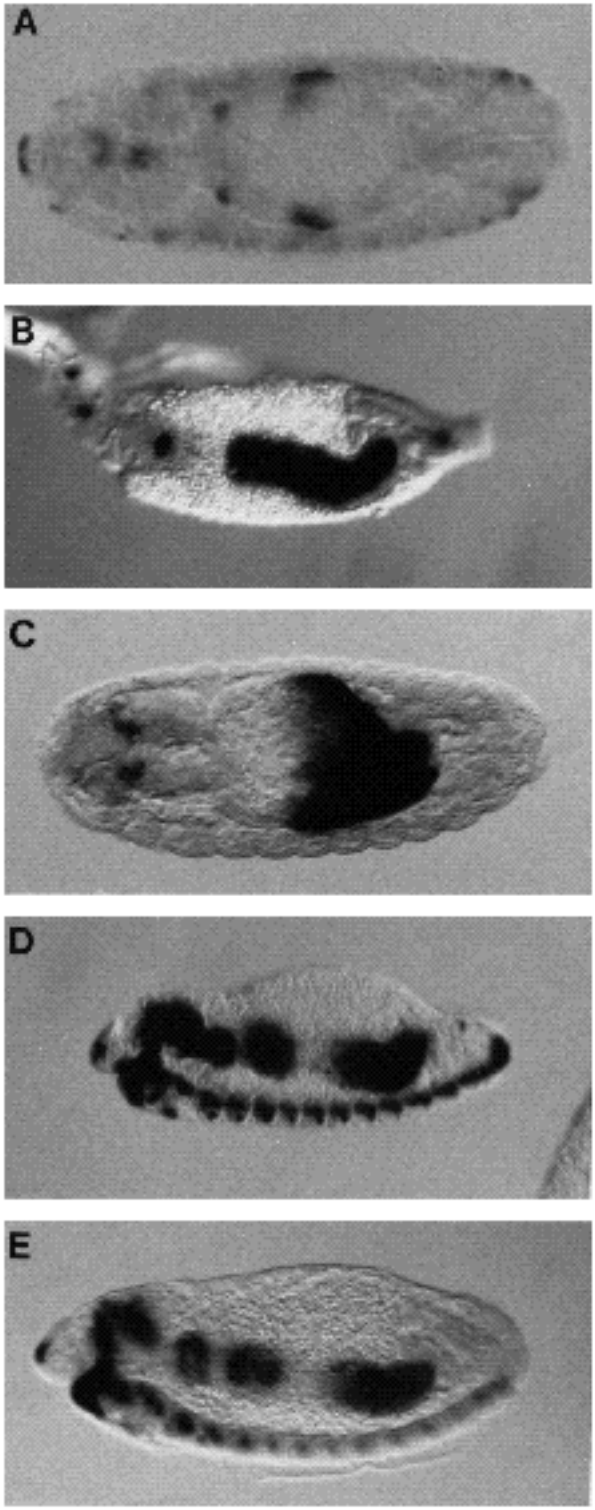

Fig. 5. *pdm-1* expression in *abd*-A and *Pc* mutants. In situ hybridizations to wild-type and mutant stage 13 embryos are shown. Embryos used were from wild type (A), *abd*-A (B-D) and *Pc* (E). Probes used were *dpp* (A,B), *lab* (C), and *pdm-1* (D,E). Pc^3 embryos were identified as described in Materials and Methods. *dpp* (B) and *lab* (C) expression clearly extend throughout most of the posterior midgut in *abd-A* mutant embryos whereas *pdm-1* repression remains limited to the central domain of the midgut (D).

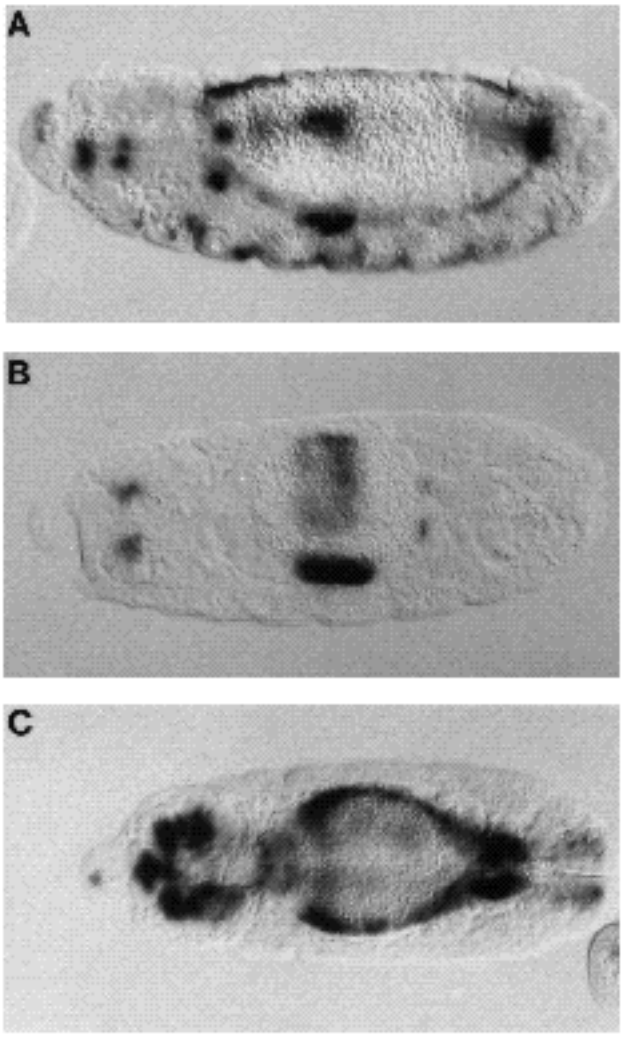

Fig. 6. *pdm-1* and *lab* regulation are uncoupled in *opa* mutant embryos. In situ hybridization to *opa* mutants. Probes used were *dpp* (A), *lab* (B) and *pdm-1* (C). *dpp* transcript distribution (A) is similar to that seen in wild-type embryos (see Fig. 5A), and *lab* is induced to high levels in *opa* mutant embryos (B). Despite the induction of *lab* in the endoderm (presumably through *dpp*), *pdm-1* is not repressed in the central domain (C). Anterior *pdm-1* repression is normal despite the absence of *Scr* expression in the VM (see Tremml and Bienz, 1989b). This is in agreement with the results shown in Fig. 2B.

below). The results of the expression studies in *abd-A* and *Pc* mutants therefore indicate that *pdm-1* and *lab* do not use the identical genetic cascade for their regulation across germ layers in the central midgut.

If *lab* and *pdm-1* use different genetic cascades for regulation across germ layers, it should be possible to identify certain mutations that uncouple *lab* and *pdm-1* regulation in the central midgut endoderm, i.e. one gene is still properly expressed while the other gene is not. Genetic alterations that interfere with the establishment of parasegmentally restricted factors in the VM might be identified among mutations in the pair-rule class of segmentation genes (see Tremml and Bienz, 1989b). We have looked at the expression patterns of *dpp, wg, lab* and *pdm-1* in all of the eight known pair-rule mutant backgrounds (data not shown). In all but one class of pair-rule mutants, *lab* and *pdm-1* regulation in the central midgut correlated with the presence or absence of *dpp* in VM parasegment 7 cells. In embryos mutant for the pair-rule locus *opa,* Tremml and Bienz (1989b) found that *Ubx* is expressed in a domain of approximately the normal width in the VM of the central midgut. We found that while *dpp* expression as well as *lab* induction were normal (Fig. 6A,B), *pdm-1* repression did not (or only very weakly) occur in the central domain in *opa* mutants (Fig. 6C). This suggests that at least one genetic component that is essential for *pdm-1* repression but dispensable for *lab* induction is not properly expressed in *opa* and provides additional and independent strong evidence for the use of a different genetic cascade for *lab* induction and *pdm-1* repression in the central part of the midgut endoderm (see Discussion).

Function of *pdm-1* during midgut development

What is the function of *pdm-1* in midgut development? No mutation that maps to the *pdm-1* gene has thus far been characterized. However, there exist two deficiencies (Df(2L)*prd*$^{1.7}$ and Df(2L)*Prl*) that remove either the entire *pdm-1* gene or part of it, respectively, and that do not show detectable *pdm-1* transcript levels at any stage during embryonic development (data not shown). Although these deficiencies remove the segmentation gene *paired* (*prd)* in addition to *pdm-1*, no detectable alterations such as defects with respect to midgut fusion, lack of *lab* induction or lack of formation of the three midgut constrictions can be observed (data not shown). This is not due to the presence of a second POU box gene in the 33F area (named *pdm-2* (Billin et al., 1991; Lloyd and Sakonju, 1991) or *dPOU-28* (Dick et al., 1991)), because this gene is not expressed in the Df(2L)*prd*$^{1.7}$ mutant embryos either (data not shown; in wild-type embryos, *pdm-2* is strongly expressed in the proventriculus and only very weakly in a pattern very similar or identical to *pdm-1* in the remaining midgut). Mutations in individual *pdm* genes will have to be analysed in detail with respect to gene regulatory defects and/or later-occurring morphological alterations in the midgut to find out whether the differentiation of the various specialized cell types found in the midgut epithelium (Filshie et al., 1971) is affected or not.

DISCUSSION

We have shown that the expression pattern of the *Drosophila* POU box gene *pdm-1* in the developing endoderm is dramatically modified upon midgut fusion. Repression in a central domain is dependent on the activity of *Ubx* and *dpp* in the adjacent VM, but *dpp* does not seem to be the signal that mediates the effect across germ layers. Repression in a more anteriorly located domain is independent of known homeotic selector genes and genes encoding secreted signalling molecules (see Fig. 7 for an overview).

Repression in the anterior domain

Although the anterior domain in which *pdm-1* is repressed

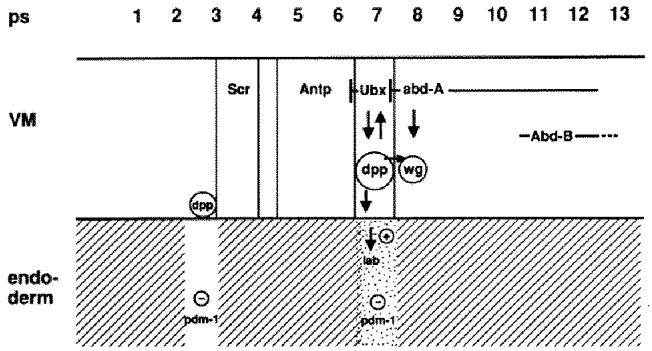

Fig. 7. Schematic representation of *pdm-1* expression. *pdm-1* expression in the endoderm of stage 13 embryos is compared to the expression domains of other components of the VM and the endoderm. Parasegments in the VM are indicated as mapped by Tremml and Bienz, 1989a. The distribution and interactions among homeotic genes in the VM are mainly from Tremml and Bienz, 1989a. Interactions between homeotic genes and genes encoding secreted molecules as well as the homeotic gene *lab* in the endoderm are taken from Immerglück et al. (1990), Reuter et al. (1990) and Panganiban et al. (1990). *pdm-1* expression in the endoderm of stage 13 embryos is indicated by the stippled area. *lab* induction occurs in the speckled area.

upon midgut fusion is located adjacent to *dpp*-expressing VM cells, repression is not dependent on *dpp* function (Fig. 3B,C). This result is consistent with the observation that dpp protein has not been found migrating into or across the endoderm at the anterior end of the midgut (Panganiban et al., 1990). In addition, repression in the anterior domain did not depend on the activity of any homeotic gene known to be expressed in the VM, either for repression per se or for positioning of the repression domain. At the moment, we do not know whether anterior repression involves interaction between germ layers or, alternatively, relies entirely on positional information present in the endoderm itself. We have investigated *pdm-1* expression in several mutant backgrounds in which the establishment of a normal VM is disrupted (see Tremml and Bienz, 1989b; Bodmer et al., 1990). In these mutants, migration of the endodermal midgut primordia is abnormal and it was not possible to investigate whether anterior repression still occurred or not. Although the concomitant occurrence of anterior and central repression upon midgut fusion as well as the distinct endodermal borders of repression argue that anterior repression is also mediated via induction, further genetic experiments will be required to demonstrate such a mechanism in the anterior midgut.

Possible functions of *Ubx* and *dpp* in central repression of *pdm-1*

The analysis of the regulation of the homeotic gene *lab* has revealed an induction process between the VM and the endoderm involving a cascade of interacting genes (Immerglück et al., 1990; Reuter et al., 1990; Panganiban et al., 1990). The observations reported here indicate that the regulation of *pdm-1* in the endoderm is mediated by a similar but not identical genetic cascade. In the case of *lab*, *Ubx* expression in VM parasegment 7 results in the activation of the *lab* gene in the adjacent endoderm whereas, in the case of *pdm-1*, *Ubx* triggers a repression of the gene in the same endodermal cells. This repression of *pdm-1* in the endoderm is not brought about by the lab protein itself, demonstrating that *pdm-1* is not simply downstream of *lab* in the same induction cascade. In addition, *pdm-1* and *lab* do not respond similarly to ectopic *Ubx/dpp* expression. This is demonstrated in Pc^3 and *abdA* mutant backgrounds where *lab* can be induced, due to ectopic *Ubx* expression, in most of the anterior and posterior midgut endoderm, respectively; in contrast, the central repression domain of *pdm-1* remains unchanged in these mutant embryos. However, it is possible that the lack of ectopic *pdm-1* repression in *abdA* and Pc^3 is due to interference from other derepressed genes (homeotic genes in the case of Pc^3). Independent and more compelling evidence supporting the conclusion that different genetic cascades are involved in the regulation of *lab* and *pdm-1* was obtained in the analysis of germ layer interactions in pair-rule mutant embryos. The lack of central *pdm-1* repression in *opa* mutants (despite the presence, in the central VM, of *Ubx* and *dpp*) contrasts the induction of *lab* in the same mutant background, demonstrating that *pdm-1* and *lab* respond differently to certain genetic alterations.

There are several hypotheses that can be put forward to explain the inability of *Ubx* and *dpp* to repress *pdm-1* outside the central midgut endoderm adjacent to VM parasegment 7. Induction processes can be subdivided into different steps, including the generation, the transmission and the interpretation of a signal. The difference(s) in the genetic cascades used in the spatial regulation of *lab* and *pdm-1* across germ layers could occur at any of these steps. In the following, our experimental results are discussed with respect to possible differences occurring at certain steps mentioned above.

Our results suggest that *dpp* might not be the only molecule that mediates the negative effect of *Ubx* on *pdm-1* across germ layers. *dpp* has been implicated in the activation and/or maintenance of *Ubx* in the VM (Panganiban et al., 1990); the absence of central *pdm-1* repression in dpp^{s4} mutant embryos could thus be indirect and due to a decrease in *Ubx* activity (or the activity of other *dpp* targets) in parasegment 7 of the VM. Several observations suggest indeed that the effect of *dpp* on *pdm-1* repression is indirect (maybe via *Ubx*) and that *dpp* is not the (only) signal responsible for *pdm-1* repression in the central domain. (1) Reuter et al. (1990) demonstrated that not all endodermal cells adjacent to VM parasegment 7 respond to *dpp* with the induction of *lab* expression; only those endodermal cell that are in direct contact with VM cells in stage 13 embryos accumulate lab protein. Even at later stages when all endodermal cells are in contact with *dpp*-expressing VM cells in parasegment 7, some cells do not express *lab*. This indicates that the *dpp* effect is very short ranged, presumably requiring cell-cell contact. In the case of *pdm-1*, however, all the cells within the central repression domain seem to be emptied of *pdm-1* transcripts in stage 13 embryos in a *Ubx/dpp*-dependent process, which might reflect the use of a different secreted signalling molecule. (2) In the anteriormost cells in which lab protein accumulates in a *dpp*-dependent pathway, *pdm-1* transcripts do not disappear.

Thus even in wild-type embryos, *pdm-1* repression does not occur in a subset of *dpp* responsive midgut cells. (3) Weak *pdm-1* repression in the central domain is still observed in *dpp* mutant embryos (see Fig. 3C), whereas inhibition of repression is virtually complete in *Ubx* mutant embryos. It is most unlikely that the residual weak *pdm-1* repression in dpp^{s4} mutant embryos is due to weak *dpp* activity, since no *dpp* RNA is detectable in the VM of these mutants (Panganiban et al., 1990; our own observation). A certain signalling capacity is therefore retained in dpp^{s4} mutants with respect to *pdm-1* repression, and this capacity might depend on the residual activity of *Ubx* (Panganiban et al., 1990). (4) The strongest arguments against direct *dpp*-mediated repression of *pdm-1* in the central domain are based on results obtained in *opa* mutant embryos. In *opa*, *dpp* is still expressed in the central region of the VM and capable of inducing *lab*; however, *dpp* is not able to mediate the negative effect on *pdm-1* expression and *pdm-1* remains active in the central midgut endoderm in *opa* mutants. A putative *opa* transcript has recently been identified and its expression pattern analysed (S. DiNardo and M. Benedyk, personal communication). Up to stage 12 of embryonic development, this transcript is not detectable in cells that will give rise to the endodermal gut epithelium (S. DiNardo and M. Benedyk, personal communication) and it is therefore unlikely that the effect of *opa* on *pdm-1* repression is exerted directly in endodermal cells. Thus, the lack of *pdm-1* repression in *opa* mutants is most likely caused by a defect in the signalling VM cells, the primordium of which resides within the expression domain of the segmentation genes such as *opa*. If, as suggested above, *opa* has no effect on the endoderm before VM cells interact with it, the presence of *dpp* in *opa* mutants (Fig. 6A) should result in *pdm-1* repression if *dpp* encoded the only mediating signal. The observed lack of *pdm-1* repression in *opa* mutants thus suggests strongly that *dpp* is not the signal (or not the only signal) that mediates the negative effect of the VM on endodermal *pdm-1* regulation in the central midgut.

Molecules with signalling capacities that are required for central *pdm-1* repression and whose function is affected by mutations in the *opa* gene might be identified among the segment-polarity genes (for a review, see Peifer and Bejsovec, 1992; Ingham and Martinez-Arias, 1992). We have tested central *pdm-1* repression in embryos lacking the functional gene products *patched (ptc), hedgehog (hh)* or *fused (fu).* In none of these mutants was *pdm-1* repression abolished (data not shown).

The interpretation that *dpp* does not encode the mediating signal in *pdm-1* regulation is also consistent with the observed inability of ectopic *dpp* (as seen in *abd-A* and *Pc* mutants) to repress *pdm-1* in the anterior and posterior midgut endoderm. In addition, we have never seen an enlargement of the repression domain towards more anterior cells by ectopic activation via heat treatment of transgenic flies carrying a *Ubx* heat-shock construct whereas the same heat-shock procedure resulted in *lab* induction (Reuter et al., 1990; our unpublished observation). Considering the use of a signalling molecule distinct from *dpp* that mediates *Ubx*-dependent silencing of *pdm-1*, we propose that either this molecule is only present (or active) in the central VM (possibly respecting parasegment 7 borders) in *abd-A* and *Pc* mutants or, alternatively, that this signal can only be translated into an appropriate nuclear response in the endoderm adjacent to VM parasegment 7. In the first case, the restricted localization of a putative novel signalling molecule to VM parasegment 7 cells in wild-type, *abd-A* and *Pc* mutant flies would require an additional factor(s) whose expression is restricted to parasegment 7 and remains restricted therein upon ectopic activation of *Ubx* and *dpp* in *abd-A* and *Pc* mutants. The parasegment 7-specific expression of this factor would therefore not be controlled by the distribution of homeotic gene products in the VM. In this scenario, both *Ubx* and such a parasegment 7-specific factor would be required for regional restriction of *pdm-1* repression in the central endoderm. In the second case, *pdm-1* repression would require a factor(s) that is prelocalized in endodermal cells adjacent to VM parasegment 7 cells. Factors can indeed be prelocalized to this particular region of the endoderm, as demonstrated by *lab* which is expressed in the tip of the extending arms during the growth of anterior and posterior midgut primordia (Mlodzik et al., 1988; Diederich et al., 1989). We have shown that *lab* itself is not required for repression but other factors localized to the same region might be necessary for the spatial restriction of *pdm-1* repression.

To test the above discussed models, which could explain certain aspects of the differences seen in the regulation of *lab* and *pdm-1* across germ layers, a genetic screen aimed at the identification of the proposed factors will have to be undertaken in order to get more insight into the genetic circuitry controlling the regulation of these genes across germ layers. Although these interactions take place relatively late during embryonic development, such a genetic screen should in principle be possible due to the largely independent development of the mesoderm and the endoderm (Lawrence and Johnston, 1984; Tremml and Bienz, 1989b).

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