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# *Drosophila* endoderm development requires a novel homeobox gene which is a target of Wingless and Dpp signalling

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**Abstract**

We have identified and cloned a novel type of homeobox gene that is composed of two homeodomains and is expressed in the *Drosophila* endoderm. Mutant analysis reveals that its activity is required at the foregut/midgut boundary for the development of the proventriculus. This organ regulates food passage from the foregut into the midgut and forms by the infolding of ectoderm and endoderm-derived tissues. The endodermal outer wall structure of the proventriculus is collapsed in the mutants leading to a failure of the ectodermal part to invaginate and build a functional multilayered organ. Lack-of-function and gain-of-function experiments show that the expression of this homeobox gene in the proventriculus endoderm is induced in response to Wingless activity emanating from the ectoderm/endoderm boundary whereas its expression in the central midgut is controlled by Dpp and Wingless signalling emanating from the overlying visceral mesoderm. 

*Keywords:* Homeobox; Proventriculus; Wingless; Dpp; Endoderm

## 1. Introduction

The generation of tissues and organs in multicellular organisms involves numerous local signalling events that are required for the determination of cell fates and morphogenesis during development (Gurdon, 1992; Greenwald and Rubin, 1992; Artavanis-Tsakonas et al., 1995). One class of molecules important for such interactions are secreted signalling molecules. Several conserved families of secreted growth and differentiation factors, with major roles in the organization of patterning, have been identified in various organisms (Greenwald and Rubin, 1992; Bürglin, 1995; Lawrence and Struhl, 1996). The *Drosophila* genes *wingless* (*wg*) and *decapentaplegic* (*dpp*), encode secreted molecules that belong to the Wnt and TGF$\beta$ families of growth factors, respectively (Padgett et al., 1987; Rijsewijk et al., 1987; Nusse and Varmus, 1992; Hogan, 1996; Massagué, 1996). Both have been studied extensively during early embryogenesis, limb and eye patterning and endoderm induction (Basler and Struhl, 1994; Bienz, 1994; DiNardo et al., 1994; Brook et al., 1996; Cohen, 1996; Lawrence and Struhl, 1996).

For endoderm development, Wg and Dpp-dependent signalling cascades emanating from the visceral mesoderm were shown to induce the formation of the second midgut constriction by regulating the expression of transcription factors expressed in the endoderm, such as Labial and D-Fos (Bienz, 1997). In the ectodermal fore- and hindgut *wg*, *dpp* and *hedgehog* (*hh*) which encodes another pattern inducing signalling molecule (Ruiz i Altaba, 1997), are not expressed in the visceral mesoderm but in the gut epithelium only. These genes were shown to coordinate morphogenesis in signalling centres which become established at the junctions of the ectodermal and endodermal tissue layers (Pankratz and Hoch, 1995; Hoch and Pankratz, 1996). At the junction of the foregut and midgut, they direct the formation of the proventriculus, a gut-associated organ mediating food passage in the larva (Snodgrass, 1935; Poulson, 1950; King, 1988; Campos-Ortega and Hartenstein, 1997). It has remained elusive, however, how the signals are transformed into local activation of target genes that induce the morphogenetic movements.

Here we describe the cloning and functional analysis of a

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novel homeobox gene which we named *compass*. The same gene has been named in a most recent publication by Nakagoshi et al. (1998) *defective proventriculus* (*dve*). For reasons of consistency we will use the latter gene designation. We present evidence that *dve* is required for epithelial morphogenesis during proventriculus development. We show that *dve* encodes two differentially spliced transcripts, the larger one coding for two homeodomains. We demonstrate by mutant analysis and misexpression studies that *dve* is activated in the proventriculus endoderm in response to Wg signalling emanating from the foregut/midgut boundary; whereas not only Dpp but also Wg signalling emanating from the visceral mesoderm induce *dve* expression in the central midgut region. Thus *dve* is a novel target gene of two major signalling pathways that coordinate gut morphogenesis in the *Drosophila* endoderm.

## 2. Results

### 2.1. Identification of the dve locus

In a search for regulators of gut development, we screened a collection of enhancer trap lines for expression patterns and mutant phenotypes in the developing organ. We identified four non-complementing enhancer trap lines, l(2)01829, l(2)01738, l(2)05084 and l(2)06432 (Karpen and Spradling, 1992) with P element insertions in the chromosomal region 58D1–2. These lines are homozygous and transheterozygous lethal in the 1st instar larval stage. To identify the cause for the lethality, we performed a feeding assay using dyed yeast (Pankratz and Hoch, 1995). We found that in homozygous larvae, food passage is blocked at the proventriculus suggesting that the lethality is due to a feeding defect (Fig. 1A–D). This occurred in larvae that were homozygous for the P insertion or transheterozygous for each combination of the P chromosomes. Higher magnification of the foregut region of the mutant larvae revealed a collapsed outer wall of the proventriculus (Fig. 1D, compare with Fig. 1B). This organ is a multilayered structure that regulates food passage from the foregut to the midgut (Strasburger, 1932; Snodgrass, 1935; Skaer, 1993; Pankratz and Hoch, 1995; Campos-Ortega and Hartenstein, 1997). In transheterozygous condition with the deficiency Df(2R) 58–5 which displays a deletion of the chromosomal bands 58B3–59A1, the P alleles also showed larval lethality. The proventriculus defect is, however, slightly more severe than the one observed in homozygous P mutants or transheterozygotes (data not shown). These results suggest that the P alleles represent strong hypomorphic alleles of a novel gene locus which we named *compass* but now refer to it as *dve* (see Section 1). We named the mutant P alleles $dve^{P1738}$, $dve^{P1829}$, $dve^{P5084}$ and $dve^{P6432}$. Subsequent mutant analysis refers to $dve^{P1738}$ if not stated otherwise (the $dve^{P1738}$ allele corresponds to $dve^{1}$ described in Nakagoshi et al., 1998).

### 2.2. dve activity is required for proventriculus development

The proventriculus is an organ which is composed of gut epithelium that is derived from two germ layers, the ectoderm and the endoderm (Skaer, 1993; Pankratz and Hoch, 1995; Campos-Ortega and Hartenstein, 1997). Proventriculus development takes place during embryogenesis and becomes overt in stage 13 embryos. Cells in the posterior part of the foregut epithelium migrate out from a mesoderm free zone to form the keyhole (Fig. 1E) (Pankratz and Hoch, 1995). The keyhole tissue then folds back on itself to generate a structure called the cardiac valve which is subsequently pushed as an extension of the oesophagus into an endodermal sac-like midgut chamber which forms the outer wall of the proventriculus (Fig. 1F) (King, 1988). The visceral mesoderm ensheathes the organ and the stomatogastric nervous system innervates the cardiac valve (Campos-Ortega and Hartenstein, 1997).

To investigate whether the proventriculus defect in *dve* mutants is established during embryogenesis, we performed anti-Fork head (Fkh) and anti-Fasciclin (Fas) III antibody stainings of wild type and *dve* mutant embryos (Fig. 1E–I). Anti-Fas III staining labels the septate junctions of the gut epithelia and anti-Fkh staining marks the ectodermal foregut cells. In *dve* mutant embryos, the specification of the proventriculus primordium proceeds as in wild type (data not shown). In stage 17 mutant embryos the endodermal outer wall structure shows a collapsed phenotype and the ingrowth of the ectodermal valve cells into the endoderm fails (Fig. 1H,I compare to Fig. 1F,G). TUNEL stainings revealed that no cell death occurs in the defective outer wall affected in the mutants (data not shown). Furthermore, BrdU experiments and cell counts indicate that the number of outer wall cells is not changed as compared to wild type (data not shown). These results suggest that *dve* might be required for cell shape changes or adhesion processes maintaining the tissue integrity of the outer wall cells.

### 2.3. Molecular characterization of the dve locus

Mobilization of the P elements in the $dve^{P1738}$, $dve^{P5084}$ and $dve^{P6432}$ flies resulted in independent excission lines that were homozygous viable and fertile demonstrating that P element insertion had caused the *dve* mutation. Similar results could not be obtained with $dve^{P1829}$, most likely due to a second P element-independent hit on the chromosome. To clone the gene affected in the *dve* locus we isolated genomic DNA flanking the P insertions by plasmid rescue (see Section 4). The DNA fragments were used to initiate a chromosomal walk in which we isolated and characterized about 50 kb of genomic DNA sequence flanking the P insertion sites (Fig. 2A). Transcribed DNA sequences in the genomic region were identified by cDNA and Northern blot analysis. We sequenced cDNAs that were obtained from three different embryonic cDNA libraries and the corresponding portions of the genomic

DNA (see Section 4). The structure of the transcription unit and the organization of the two differentially spliced transcripts of 1.7 kb and 5.0 kb in length are summarized in Fig. 2A. The two transcripts share the first two exons and differ in their 3′ region which display alternating polyadenylation sites recovered in the corresponding classes of cDNAs. The short transcript contains 1 and the long transcript 5 introns, respectively (Fig. 2A). Northern blot analysis revealed strong expression of the 5.0 kb transcript and weaker expression of the 1.7 kb transcript in embryos

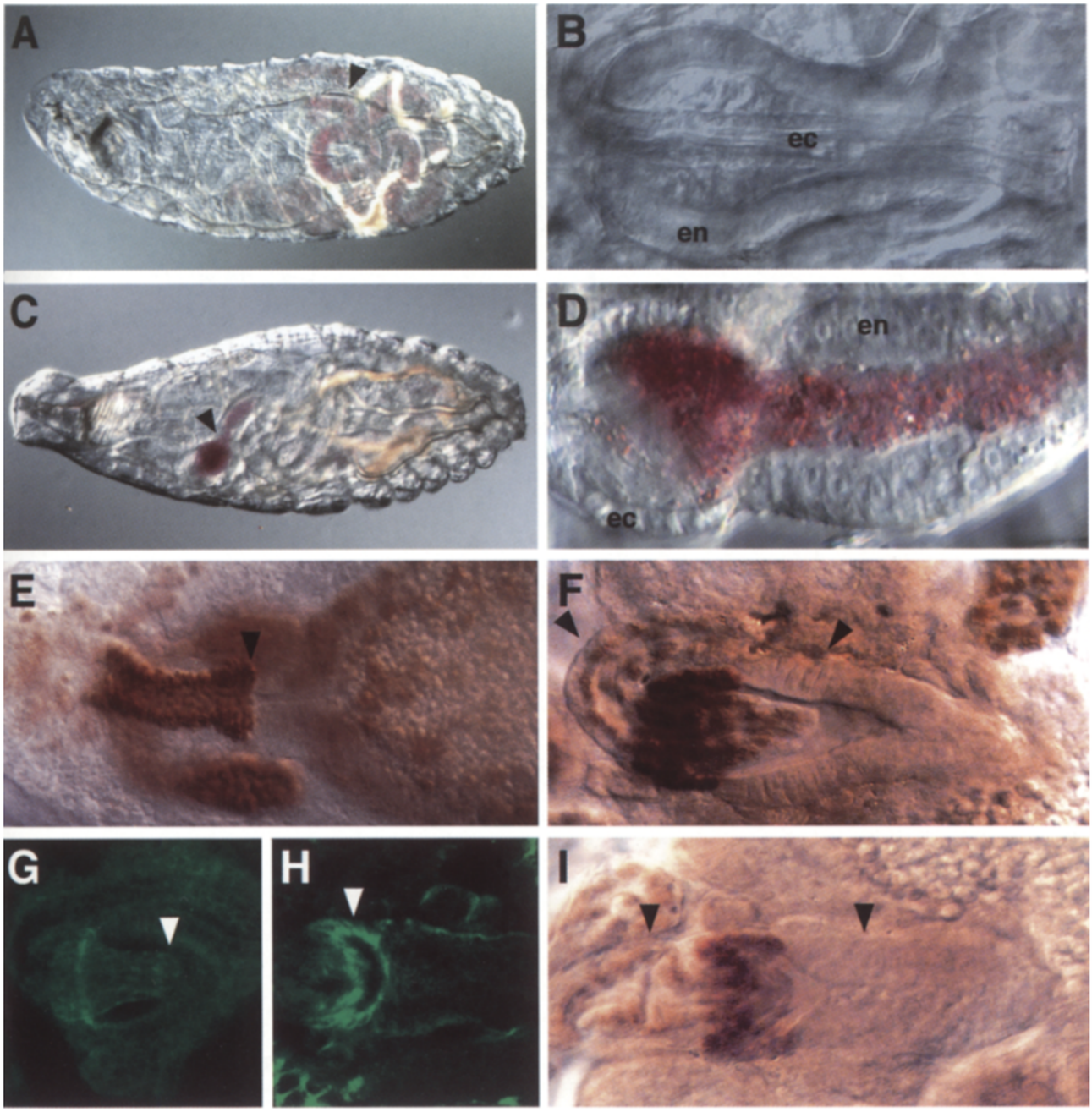


Fig. 1. *dve* mutants display a defective proventriculus. A feeding assay using dyed yeast, reveals that in a wild type 1st instar larva (A), the food is mostly found in the midgut (arrow), whereas food passage is blocked at the proventriculus (arrow) in a *dve* mutant 1st instar larva (C). The proventriculi of the wild type and the *dve* mutant larva in (A) and (C) is shown in a magnification in (B) and (D), respectively. Note that the ectoderm (ec) cells fail to migrate into the collapsed endoderm (en) of the proventriculus in the *dve* mutant larva. (E,F,I) Anti-Fkh antibody staining and (G,H) anti-FasIII antibody staining marking the septate junctions of epithelial cells (see text). (E) In wild type stage 13 embryos, prior to keyhole formation, Fkh expression marks the ectodermal foregut cells. The arrow indicates the boundary of ectoderm and endoderm. In stage 17 wild type embryos (F) the ectodermal oesophagus cells (left arrow) migrate into the proventriculus endoderm forming the outer wall structure (right arrow). In a stage 17 *dve* mutant embryo (I), the outer wall of the proventriculus is collapsed (right arrow) and the foregut ectoderm fails to invaginate and forms a protrusion in the oesophagus (left arrow). Anti-FasIII staining in a wild type stage 17 embryo (G) and a stage 17 *dve* mutant embryo (H) visualizes the cardiac arrest phenotype (Pankratz and Hoch, 1995) of the ectodermal cells (compare arrow in (G) and (H)) that contain high levels of septate junctions and fail to migrate into the endoderm part of the organ.

(Fig. 2B). Sequence analysis of the plasmid rescue fragments allowed the localization of the P elements in respect to the transcription unit. The P element of *dve*$^{P1829}$ is inserted upstream of the putative transcription start site

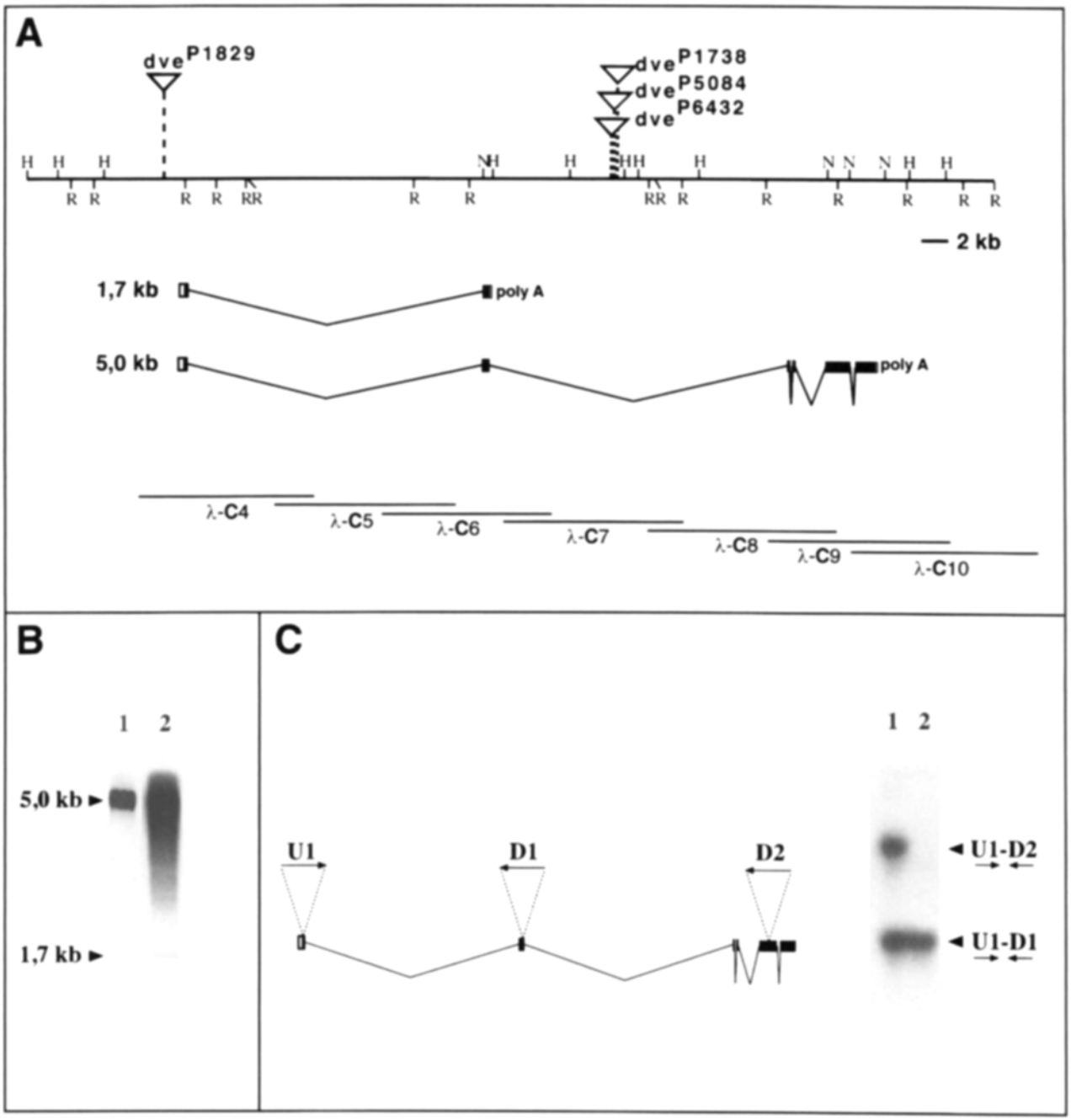


Fig. 2. Molecular organization of the *dve* locus. (A) Physical map of the genomic region containing the *dve* gene. The genomic region was characterized by mapping, genomic λ-phages (bottom) and a 59.5 kb genomic P1 clone. The P element insertion sites of the four enhancer trap lines *dve*$^{P1829}$, *dve*$^{P1738}$, *dve*$^{P5084}$ and *dve*$^{P6432}$ are indicated on top. The genomic organization of the *dve* transcription unit which contains two transcripts of 1.7 kb and 5.0 kb in length was established by sequencing the cDNAs and the corresponding genomic DNA including the exon/intron boundaries (see Section 4). The 5.0 kb transcript is encoded by 6 exons, the 1.7 kb transcript contains 2 exons, which are colinear with the 5′ portion of the longer transcript. Open bar: untranslated leader and trailer region; black bar: longest open reading frame. H, HindIII; N, NotI; R, EcoRI. (B) Autoradiogram of a Northern blot with poly A( + ) RNA from 0 to 18 h embryos hybridized with a $^{32}$P-labelled *dve* cDNA containing the sequence of the 5.0 kb transcript. The cDNA detects a strong signal of 5.0 kb and a weak signal of 1.7 kb as deduced from comparison to an RNA molecular size marker. Lane 1 represents 5 μg RNA loading and in lane 2, 20 μg RNA were loaded. We cannot exclude the presence of more *dve* transcripts that are weakly expressed. (C) RT-PCR approach using poly A( + ) RNA of wild type (lane 1) and *dve* mutant larvae (lane 2) that were scored using the feeding assay. In conjunction with an upstream primer (U1), we used one downstream oligonucleotide (D1), occurring in both transcripts and a second downstream oligonucleotide (D2) localizing to exon 5 of the 5.0 kb transcript, respectively. Fragments corresponding to both the 1.7 kb and the 5.0 kb transcripts can be detected in 1st instar larvae of wild type animals (lane 1). A fragment corresponding to the 3′ portion of the 5.0 kb transcript which contains the homeoboxes is, however, not detectable in the *dve* mutant larvae (lane 2).

whereas the other three P elements are inserted in the second intron (Fig. 2A).

In order to test whether the characterized transcripts in the *dve* locus are affected in the *dve* mutants, we performed RT-PCR experiments on total RNA of wild type and $dve^{P1738}$ mutant larvae that were scored using the feeding assay (Fig. 2C). Transcript-specific primers allowed the amplification of characteristic PCR product of the *dve* mRNAs (Fig. 2C). In wild type larvae, PCR products could be obtained that suggest that both the 1.7 kb transcript and the 5.0 kb transcript are present. In *dve* mutant larvae a PCR product characteristic for the 3′ portion of the 5.0 kb transcript could not be obtained. This provides evidence that the 5.0 kb transcript might carry the *dve* function during proventriculus development. Several other lines of evidence further argue in this direction. The 5.0 kb transcript is expressed in the endodermal proventriculus cells that are affected in *dve* mutants (see below). RNA in situ hybridization reveals the absence of the 5.0 kb transcript in *dve* mutant embryos (see below). And finally, experiments in which the cDNA that corresponds to the 5.0 kb transcript was cloned into a UAS vector and expressed in the proventriculus of *dve* mutant embryos using a midgut Gal4 driver line led to a complete rescue of the proventriculus phenotype (see below). Therefore, we conclude that the transcription unit corresponds to the *dve* gene and that the 5.0 kb transcript carries the *dve* function that is required for proventriculus development.

### 2.4. dve encodes a putative homeodomain protein

The 5.0 kb *dve* transcript encodes a single open reading frame of 3060 bp which codes for a putative Dve protein of 1020 amino acids (aa) (Fig. 3A). Dve contains two homeodomain DNA-binding motifs in its C-terminal region (Bürglin, 1995; Gehring et al., 1996). Sequence alignment reveals that the two homeodomains HD1 and HD2 are homologous to each other and to the two homeodomains of a putative *C. elegans* protein encoded by the cDNA yk117e1.5 (Fig. 3B). Most notable is an unusual insertion of 10 amino acids between helices II and III of these homeodomains which puts the Dve and the *C. elegans* protein into a novel subgroup of homeodomain transcription factors. The homeodomain of the *Drosophila* Orthodenticle protein displays high similarity to the Dve and yk117e1.5 homeodomains in helix III (Fig. 3B). Outside the homeodomains, the Dve and the *C. elegans* protein share a region of sequence similarity with the human SATB-1 protein (Fig. 3C,D) which has been shown to function as a matrix-associated DNA-binding factor (Dickinson et al., 1992). We will denote this putative protein domain as the Compass domain (Fig. 3C).

The 1.7 kb *dve* transcript and the 5.0 kb transcript share the first two exons (Fig. 3A). The 1.7 kb transcript encodes, however, two amino acids and a trailer region which are specific for this transcript (Fig. 3A) providing strong evidence (together with the Northern blot data, Fig. 2B) that the 1.7 kb form is a bonafide transcript and not a background noise of the RNA processing machinery. This transcript encodes a single open reading frame of 735 bp that gives rise to a putative protein of 245 aa. We will designate this truncated protein version as Dve-s (Fig. 3A,D). The two homeodomains and the Compass domain are not contained within this truncated protein variant. However, Dve-s and Dve share stretches of glutamine residues which are commonly involved in protein-protein interactions (Courey and Tjian, 1988) and a PEST domain (Rechsteiner and Rogers, 1996) in their common N-terminal region (Fig. 3D).

### 2.5. dve expression patterns during embryogenesis

Expression of *dve* was examined by RNA in situ hybridization to wild type embryos. Using a digoxygenin-labelled antisense probe which detects both the 1.7 kb and the 5.0 kb transcripts showed an identical pattern to the one we obtained when using a specific probe for the 5.0 kb transcript (not shown). This indicates that the 1.7 kb and the 5.0 kb transcripts are expressed in an overlapping pattern during embryogenesis. Most prominently, *dve* expression can be found in the developing midgut endoderm (Fig. 4). Its expression can be first detected at the rostral tip of the anterior midgut primordium (Fig. 4A). This expression persists until late stages of embryogenesis and becomes confined to the outer endodermal wall of the developing proventriculus. In stage 12 embryos, *dve* is expressed in the migrating posterior midgut primordium and soon thereafter in an endodermal domain at the junction of the midgut and hindgut (Fig. 4B). In stage 13 embryos, when the anterior and the posterior midgut primordia have fused, *dve* expression is most prominent in the anterior, central and posterior portion of the midgut (Fig. 4C). Weak expression is detectable in the region from where the gastric ceacae will bud out. In stage 14 embryos, the central expression domain broadens (Fig. 4D) and covers finally the second and third midgut lobes in stage 16 (Fig. 4E) and stage 17 embryos (Fig. 4F). Additional expression domains of *dve* include the tip cells of the Malpighian tubules (not shown), mesectodermal cells (Fig. 4A), nerve cells of the central and peripheral nervous system (Fig. 4C–E) and a group of cells that lie below the pharynx (Fig. 4C,D).

### 2.6. dve activity is required in the endodermal proventriculus cells

In order to investigate whether the P element insertion in the *dve* mutants affect the transcription of the *dve* gene during embryogenesis, we performed RNA in situ hybridizations on *dve* mutant embryos with *dve* antisense probes (Fig. 5A,B). In these experiments each probe was used in combination with an *orthopedia* probe that served as an internal staining control. *orthopedia* is predominantly expressed in the developing hindgut and the anal pads

(Simeone et al., 1994). Using a 5′ probe of *dve* that detects both the 1.7 kb transcript and the 5.0 kb transcript revealed normal expression in homozygous *dve* mutants (Fig. 5A). When we used a 3′ probe specific for the 5.0 kb transcript, expression of *dve* was absent (Fig. 5B). These results suggest that the 1.7 kb transcript is still present whereas the 5.0 kb transcript which carries the homeoboxes is absent in *dve* mutants. This further indicates that the *dve* function during proventriculus development resides within the 5.0 kb transcript and is consistent with the RT-PCR experiments on *dve* mutant larvae (Fig. 2C).

As shown above, *dve* is expressed in the precursors of the

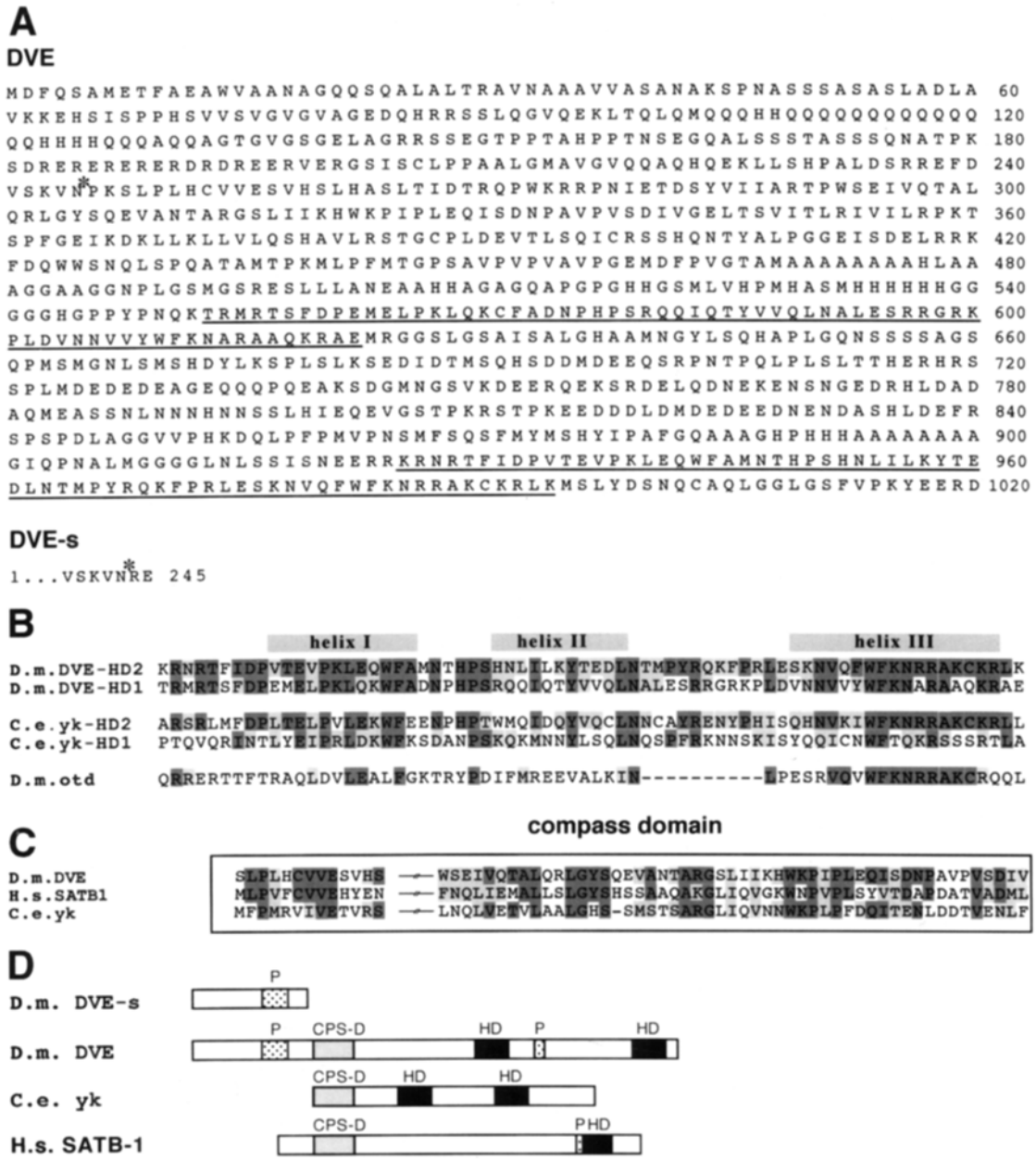


Fig. 3. The putative Dve proteins. (A) Deduced amino acid (aa) sequence of the longest open reading frame of the 5.0 kb transcript giving rise to a 1020 aa Dve protein (top; accession no.: AJ011671). The two homeodomains in the C-terminal region are underlined. The deduced aa sequence of the truncated DVE-s protein is shown below. Note that Dve and Dve-s share identical aa in the N-terminal region up to the aa residue marked by the *. (B) Alignment of the aa sequence of homeodomain HD2 of Dve with HD1 of Dve, two HDs encoded by the cDNA yk117e1.5 of *C. elegans*, and the HD encoded by the *Drosophila* gene *otd* (accession no.: AJ011672). Note the 10 aa insertion between helices II and III of the Dve and the yk117e1.5 protein. HD2 of DVE displays 65.7% similarity to HD1 of DVE, however, 75.7% similarity to HD2 of the yk117e1.5 protein. The HD2 of DVE and the HD of Otd show 64.7% identity in helix III and share a lysine at positions 60 and 50, respectively that might determine the DNA-binding specificity. Light grey boxes highlight conserved aa residues and dark grey boxes mark identical aa residues. (C) Alignment of the aa sequence of the Compass domain with the corresponding regions in the yk117e1.5 protein and human SATB1 (Dickinson et al., 1992). Dve shares 62.5% similarity with the SATB1 protein and 58.3% with the yk117e1.5 protein in this domain. Light grey boxes highlight conserved aa residues and dark grey boxes mark identical aa residues. (D) Diagram showing structural features of the predicted Dve and yk117e1.5 proteins from *Drosophila* and C. *elegans*, respectively and the human SATB1-protein. HD, homeodomain; P, PEST domain.

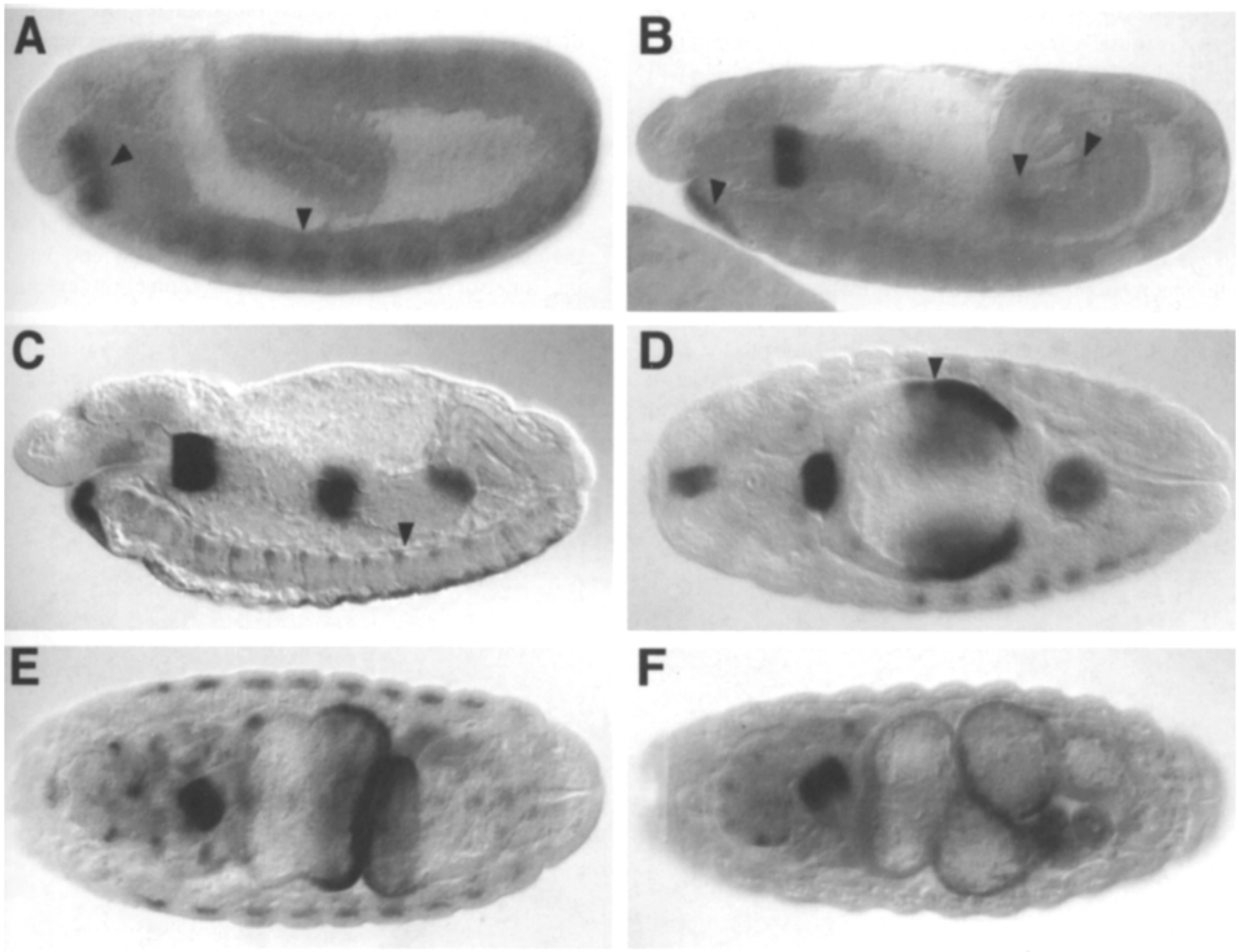


Fig. 4. Expression of *dve* in wild type embryos as revealed by RNA in situ hybridization to whole mount embryos using a digoxygenin-labelled antisense probe specific to the 5.0 kb *dve* transcript. (A) In stage 9 embryos *dve* is expressed in the anterior midgut primordium in a region giving rise to the proventriculus endoderm (left arrow). This expression domain persists throughout embryogenesis (B–F) and eventually localizes to the endoderm-derived outer wall of the proventriculus. *dve* is also expressed in mesectoderm cells (right arrow). (B) In stage 12 embryos further *dve* expression domains can be detected at the anterior tip of the posterior midgut primordium (central arrow) and in endodermal cells at the midgut/hindgut boundary (right arrow). *dve* is strongly expressed in cells which lie at the base of the pharynx (left arrow). (C) In stage 13 embryos, *dve* expression is most prominent in the anterior proventriculus primordium, in the central midgut and at the midgut/hindgut boundary. Further expression domains include neural cells in the ventral nerve cord (arrow) and the brain lobes. (D) In stage 14 embryos, *dve* expression in the central midgut extends anteriorly and posteriorly to the region where of the midgut primordia have fused (arrow). (E) In stages 16 and 17 (F) embryos, *dve* expression in the central midgut covers the second and third midgut lobes.

endodermal outer wall of the proventriculus which is collapsed in *dve* mutants (Fig. 1D). In order to see whether *dve* provides the outer wall cells with the ability to adopt a normal morphology, we performed a tissue-specific rescue experiment in *dve* mutants (Fig. 5C–F) using the UAS/Gal4 binary expression system (Brand and Perrimon, 1993). We constructed UAS-Dve transgenic fly lines using the cDNA corresponding to the 5.0 kb *dve* transcript (see Section 4). To express *dve* in endodermal cells, we used the driver line 14–3Fkh-Gal4 which mediates strong Gal4 expression in the anterior and posterior part of the midgut and the hindgut (S. Stein and M. Hoch, unpublished data). UAS/Gal4-mediated expression of Dve in the proventriculus of *dve* mutant embryos led to a complete rescue of the mutant phenotype (Fig. 5C,D,F, compare with Fig. 5E). The integrity of the outer wall cells was established again and the cardiac valve precursor was able to fully invaginate into the endoderm part of the proventriculus (Fig. 5E,F). These results demonstrate that the characterized transcription unit in the *dve* locus corresponds to the *dve* gene.

### 2.7. *dve is induced by Wg at the ectoderm/endoderm boundaries in the gut*

Morphogenetic cell movements of the proventriculus epithelium depend on the activities of the genes *hh*, *wg* and *dpp* which are expressed in the foregut (Pankratz and Hoch, 1995). These genes define through their restricted expression a signalling centre at the boundary of the foregut ectoderm and the midgut endoderm where proventriculus morphogenesis occurs. Anti-Fkh antibody stainings indicate that this boundary becomes established at the posterior mar-

gin of the keyhole structure (Pankratz and Hoch, 1995) (Fig. 1E). *wg* is initially transcribed in an expression domain that includes the ectodermal region from which the keyhole will form and extends slightly beyond it into the proventriculus endoderm (Fig. 6A,J).

The striped *dve* expression domain extends from the ectoderm/endoderm boundary towards posterior and overlaps at its anterior margin with the *wg* expression domain (Fig. 6A,J). With the onset of keyhole formation, the *wg* expression domain becomes split into two domains, one lies at the anterior border of the keyhole in the ectodermal foregut cells and the other in endodermal cells posterior to the keyhole (Fig. 6B,J). Both expression domains of *wg* persist in the developing proventriculus until very late stages of embryogenesis. *dve* expression overlaps the posterior domain of *wg* also until the end of embryogenesis (Fig. 6B,J).

In *dve* mutants, *hh* and *dpp* expression are not changed and we find no alteration of *wg* expression prior to keyhole formation (data not shown). However, after keyhole establishment is complete and the *wg* expression domain have split, the posterior expression domain of *wg* fails to be maintained. It becomes gradually weaker as compared to the anterior domain and a strong reduction eventually becomes overt in late stage 15 *dve* mutant embryos (Fig. 6C,D). This indicates that *dve* is directly or indirectly required for the maintenance of *wg* expression in the proventriculus endoderm.

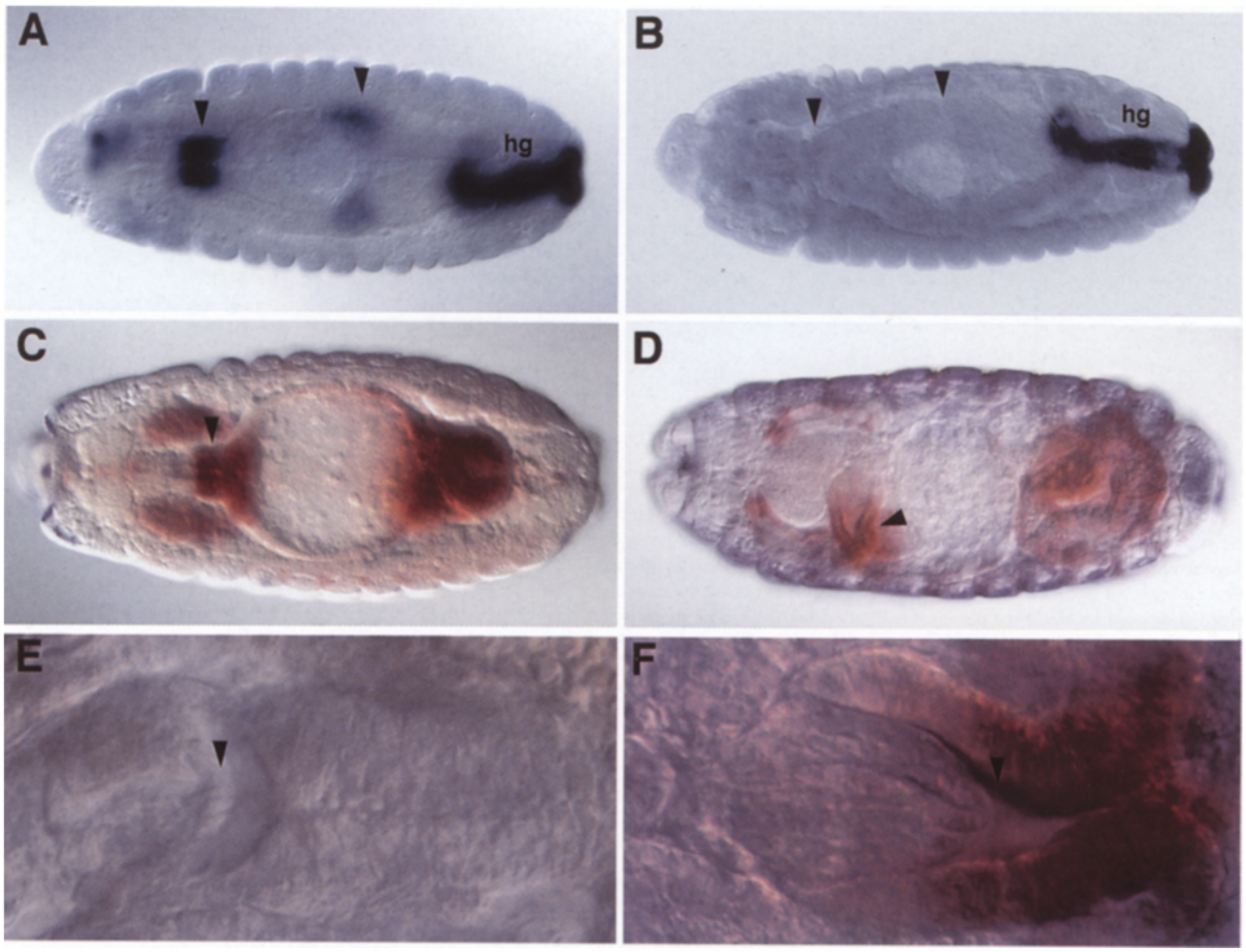


Fig. 5. *dve* is required for proventriculus development. RNA in situ hybridization experiments on whole mount embryos. (A) *dve* mutant embryo of stage 13. A *dve* probe covering the common 5′ region of both the 1.7 kb and 5.0 kb transcripts was used in combination with an *orthopedia* probe that served as an internal staining control. *orthopedia* is predominantly expressed in the developing hindgut (hg) and the anal pads (Simeone et al., 1994). Note the *dve* expression pattern in the midgut (arrows). (B) Late stage 13 *dve* mutant embryo that was incubated with a specific 3′ probe detecting the 5.0 kb transcript only, in combination with the *orthopedia* probe. Note the absence of the midgut staining pattern in *dve* mutants (arrows). (C) Stage 14 *dve* mutant embryo showing ectopic *dve* expression (red) which was induced using a UAS-Dve effector line (the transgene contains the coding sequences of the 5.0 kb *dve* transcript) and the driver line 14–3Fkh-Gal4. Ectopic *dve* expression occurs in the endodermal proventriculus cells in the anterior midgut, in the posterior midgut, the hindgut and the salivary glands. This ectopic expression of *dve* in the *dve* mutants leads in stage 17 embryos (D) to the complete rescue of the *dve* mutant proventriculus defect. For comparison, (E) is a magnification of a *dve* mutant proventriculus and (F) a magnification of the rescued proventriculus obtained upon expression of Dve in *dve* mutants. Note the stalled ectodermal cells (arrow) and the collapsed endodermal wall in the *dve* mutant proventriculus in (E) and the rescue of both of these phenotypes (arrow) in (F).

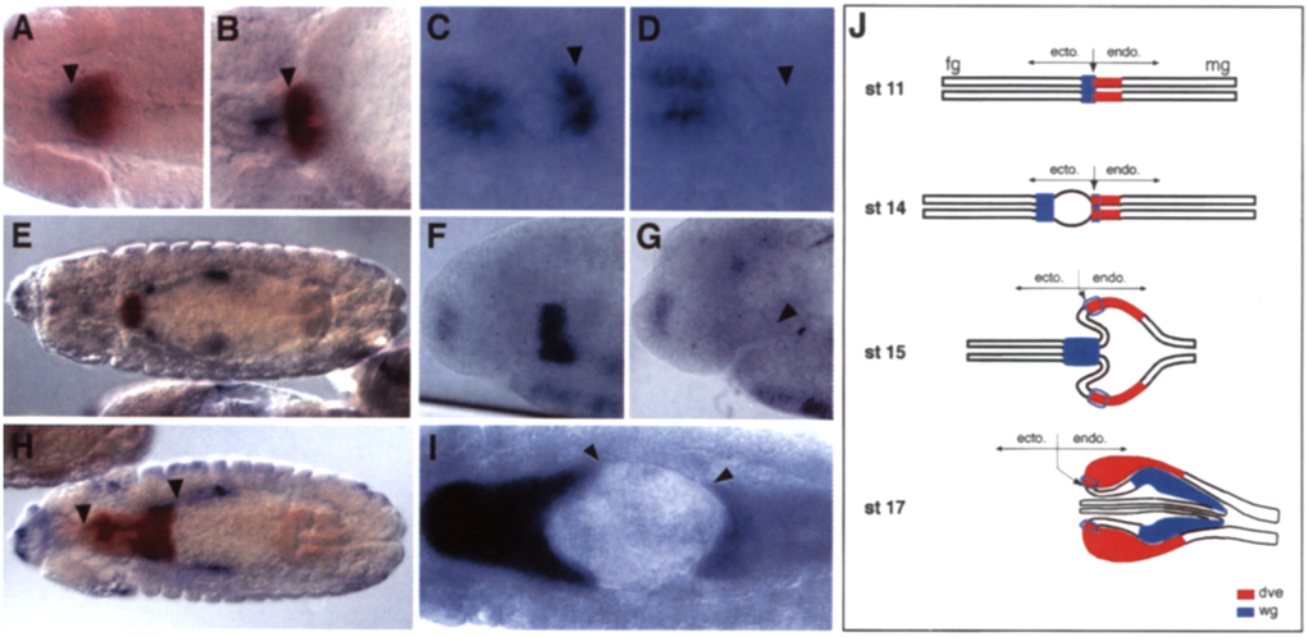


Fig. 6. Wg signalling-dependent activation of *dve* at the ectoderm-endoderm boundaries of the gut. (A–I) RNA in situ hybridizations to whole mount embryos. To monitor *dve* expression, we used a digoxygenin-labelled antisense probe specific to the 5.0 kb *dve* transcript. (A–I) RNA in situ hybridizations to whole mount embryos. (A,B) Double staining of *wg* (blue) and *dve* (red). Whereas in (A) the *wg* domain is still homogeneous in stage 11 wild type embryos, it becomes split with the onset of keyhole formation in stage 13 (B) into an anterior and a posterior subdomain. *dve* expression overlaps with the *wg* expression domain. (C) Early stage 15 wild type embryo showing that the splitting of the *wg* domain has occurred. (D) Stage 15 *dve* mutant embryo. Note that the posterior *wg* domain is strongly reduced indicating that *dve* is required for the maintenance of *wg* in this domain (compare arrows in (C) and (D)). The lack of *wg* and *dve* expression leads to morphological defects in the posterior part of the keyhole (arrow in (D)). (E) Wild type stage 13 embryo showing expression of *dve* (red) in the gut endoderm and of *dpp* (blue) in the visceral mesoderm of parasegments 3 (anterior) and 7 (middle). (F) Expression of *dve* in the anterior midgut primordium is normal in the $dpp^{BC86}$ mutant (compare to Fig. 4A). (G) Anterior expression of *dve* is absent in amorphic $wg^{IG22}$ mutant embryos (arrow). (H) Ectopic expression of Wg in the visceral mesoderm using UAS-Wg and twist-Gal4 leads to an expansion of the anterior *dve* domain (red) to both sides, into the foregut and into the midgut region (arrows). Note that the expression of *dpp* (blue) changes in the visceral mesoderm of the midgut (Staehling-Hampton and Hoffmann, 1994) but *dpp* is not expanded into the foregut nor into the region posterior to parasegment 7. The expression domain of *dve* at the midgut/hindgut boundary is out of focus in (H) but is shown in (I). Posterior *dve* expression becomes also expanded in stage 13 embryos upon ubiquitous expression of Wg throughout the visceral mesoderm. (J) Schematic representation of the expression patterns of *wg* (blue) and *dve* (red) during proventriculus development at the ectoderm/endoderm boundary of the foregut (fg) and the midgut (mg). st, stage.

To analyze whether the initiation of *dve* expression in the proventriculus primordium depends on the activity of *hh*, *dpp* or *wg*, we performed RNA in situ hybridizations with a *dve* antisense probe in the corresponding amorphic mutants. Whereas *dve* expression in the developing proventriculus was not affected in *hh* and *dpp* mutant embryos (Fig. 6F; data not shown), we found a lack of this expression domain in *wg* mutant embryos (Fig. 6G). *dve* expression was also lacking in null mutants for *armadillo* which is a downstream component of the Wg signalling pathway (Cox and Peifer, 1998; data not shown). To further examine the dependence of *dve* expression on *wg* activity, we performed ectopic expression experiments using an UAS-Wg effector line and a twist-Gal4 driver line that mediates ubiquitous expression in the visceral mesoderm (see Section 4). Upon misexpression of Wg in the entire visceral mesoderm, the *dve* expression domain expanded towards the anterior into the foregut region and towards the posterior into the midgut endoderm (Fig. 6H, compare with Fig. 6E). In the visceral mesoderm overlying the midgut portion of these embryos, also the endogenous *dpp* expression domain expands and covers the region of parasegments 2–7 (Fig. 6H) (Yu et al., 1996). However, upon ubiquitous expression of Dpp in the visceral mesoderm using UAS-Dpp transgenes in combination with twist-Gal4 we could not find a similar alteration of the anterior *dve* expression domain (compare Fig. 6H and Fig. 7H). In these embryos, the anterior *dve* domain is unchanged and does not expand into the midgut nor into the foregut (however the central expression domain of *dve* is altered due the Dpp requirement in this region, see below). Further evidence supporting the role of Wg in activating *dve* expression comes from the observation that ubiquitous expression of Wg in the visceral mesoderm also led to an expansion of the posterior *dve* expression domain (Fig. 6I) that lies at the endoderm/ectoderm boundary of the midgut and the hindgut (this domain is located in a complementary position to the one in the proventriculus). In wild type embryos, no *dpp* activity is observed in this region and ectopic Wg expression in the visceral mesoderm does not induce *dpp* expression posterior to parasegment 7 (Fig. 6H)

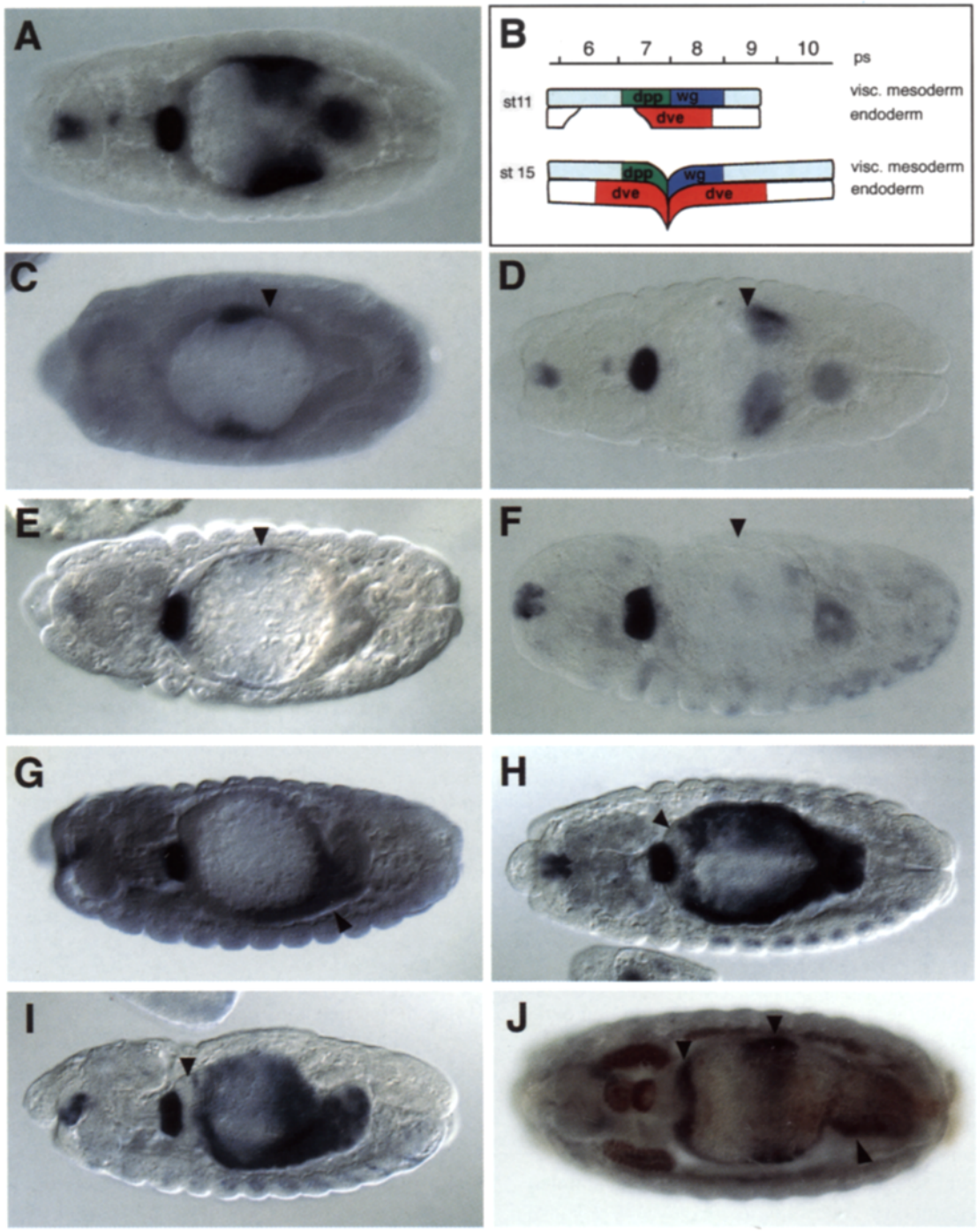


Fig. 7. Dpp and Wg signalling induce *dve* expression in the central midgut. (A,C–I) RNA in situ hybridization using a digoxygenin-labelled antisense probe specific to the 5.0 kb *dve* transcript. (J) Anti-Fkh (brown), Anti-Lab (blue) double staining. (A) *dve* expression in a stage 14 wild type embryo. Note the central expression domain covering the region below parasegments 6–9 of the visceral mesoderm. (B) Schematic representation of the dynamics of the central *dve* expression domain in relationship to *dpp* and *wg* expression. (C) *dve* expression in the central midgut is reduced at its posterior margin (arrow) in a stage 14 $wg^{CX4}$ mutant embryos. (D) In stage 14 $dpp^{S4}$ mutant embryos, *dve* expression is strongly reduced in the endoderm underlying parasegment 7 in which *dpp* is expressed in wild type (see (B)). (E) In mutants for the Dpp receptor Tkv, in which both *dpp* and *wg* expression the viseral mesoderm are absent (Affolter et al., 1994), *dve* expression is strongly reduced in the central midgut (arrow). Similarly, we find no *dve* expression in stage 14 *shn* mutant embryos (F). (G) In stage 14 *abdA* mutant embryos, *dve* is expressed throughout the posterior midgut. (H) Ectopic expression of Dpp in the visceral mesoderm using UAS-Dpp and twist-Gal4 leads to ectopic expression o *dve* in the entire midgut (stage 13 embryo). Note that only an endodermal region below parasegment 2 remains free of *dve* expression. (I) A similar ectopic induction of *dve* as in (H) was observed, when a dominant active form of the Dpp receptor $Tkv^{QD}$ was expressed in the entire midgut endoderm using UAS-$Tkv^{OD}$ and XB30Fkh-Gal4 (stage 14 embryo). (J) Stage 15 embryo, in which Dve was ubiquitously expressed in the endoderm using UAS-Dve and XB30Fkh-Gal4. Ectopic Lab expression (blue) is observed in the midgut endoderm (arrows) and presumably leads to the aberrant morphology.

(Yu et al., 1996). Therefore, a participation of Dpp signalling for the activation of the posterior *dve* domain is unlikely. The posterior *dve* domain is absent in *wg* mutants as well (not shown) further supporting the view that this domain is regulated similarly to the anterior domain. This observation fits well with previous findings that foregut and hindgut development are remarkably similar (Skaer, 1993; Pankratz and Hoch, 1995; Campos-Ortega and Hartenstein, 1997). In summary, these results provide evidence that *dve* expression at both ectoderm/endoderm boundaries in the gut is induced by Wg signalling emanating from the ectodermal fore- and hindgut, respectively (Fig. 6J).

### 2.8. Central endoderm expression of dve is controlled by Dpp and Wg signalling

In the central region of the gut endoderm, *dve* is expressed in an area in which the second midgut constriction forms (Fig. 7A,B). This structure arises in response to a signalling cascade emanating from the overlying visceral mesoderm (Skaer, 1993; Bienz, 1997). *dpp* which is induced in parasegment 7 of the visceral mesoderm by the homeodomain protein Ultrabithorax (Ubx), has been shown to stimulate the expression of Wg in the neighbouring cells of parasegment 8; Wg in turn, feeds back to parasegment 7 to stimulate Ubx expression thus establishing an autoregulatory feedback loop in the visceral mesoderm. Dpp then signals from the visceral mesoderm to the endoderm and controls together with Wg the expression of the homeotic gene *labial* (*lab*) (Immerglück et al., 1990; Panganiban et al., 1990; Reuter et al., 1990). It has been shown that high levels of Wg repress *lab* expression whereas low levels activate it (Immerglück et al., 1990; Tremml and Bienz, 1992; Hoppler and Bienz, 1995).

*dve* is expressed in a dynamic pattern in the central expression domain (Fig. 4A, Fig. 7A,B). When the anterior and posterior midgut primordia fuse, *dve* is expressed in the endoderm region that underlies parasegments 7 and 8 of the visceral mesoderm in which *dpp* and *wg* are expressed (Immerglück et al., 1990; Panganiban et al., 1990; Reuter et al., 1990) (Fig. 7A). Once the second midgut constriction starts to build at the border of parasegments 7 and 8, *dve* expression expands towards both sides into the endoderm region underlying the parasegments 6–9 (Fig. 7A,B). After the formation of the second constriction, *dve* expression persists in the second and third midgut lobes (Fig. 4E).

To study how central midgut expression of *dve* is controlled we analyzed the *dve* expression pattern in mutants affecting Dpp and Wg signalling. In *wg* mutants, *dve* was still expressed in the central region of the embryo but the expression did only weakly expand towards the posterior, as compared to wild type embryos (Fig. 7C). In *dpp* mutants we found a lack of expression in the anterior region of the *dve* domain below parasegment 7 of the visceral mesoderm where *dpp* is expressed in wild type embryos (Fig. 7D). At later stages, we found *dve* expression only in the third midgut lobe instead of the second and third lobe which displays *dve* expression in wild type embryos. In mutants for the Dpp receptor Thick veins (Tkv) (Affolter et al., 1994; Nellen et al., 1994; Penton et al., 1994) *dve* expression is strongly reduced (Fig. 7E). Similarly, we found no expression of *dve* in mutants for *schnurri* (*shn*) (Fig. 7F) which encodes a transcription factor mediating Dpp signalling (Arora et al., 1995; Grieder et al., 1995). However, in both *tkv* and *shn* mutants not only Dpp signalling but also *wg* expression is absent (Affolter et al., 1994; Grieder et al., 1995). Since *dve* is still expressed in *dpp* mutants, this suggests that also *wg* might be required for *dve* activation. In *abdA* mutants, *wg* expression is abolished and the *dpp* expression domain is expanded towards the posterior resulting in a failure of the third constriction to form (Immerglück et al., 1990; Panganiban et al., 1990; Reuter et al., 1990). In such mutants, the *dve* domain is expanded towards the posterior (Fig. 7G). This indicates that Dpp can activate *dve* in the absence of *wg* expression and that it is not the combination of both which is critical for *dve* induction. To further study the role of Dpp in activating central *dve* expression we performed mis-expression studies using UAS-Dpp and twist-Gal4 lines. In embryos where Dpp was ubiquitously expressed in the visceral mesoderm, *dve* expression was in turn ectopically activated (Fig. 7H). Only a small area around the primordia of the gastric caecae stayed free of *dve* expression. When expressing a constitutively active form of Tkv in the endoderm using UAS-Tkv$^{QD}$ and the driver line XB30Fkh-Gal4 (see Section 4), we again obtained almost ubiquitous activation of *dve* expression (Fig. 7I). Ubiquitous expression of Wg in the visceral mesoderm leads to a repression of *dve* in the central region of the embryo (Fig. 6I). These results provide evidence that the Dpp signalling pathway emanating from the visceral mesoderm plays a pivotal role in activating central *dve* expression. The mutant analysis suggests that Wg is required to maintain *dve* in the posterior part of the central *dve* domain. This interaction might be dosage-sensitive since high levels of Wg provided in the ectopic expression experiment led to the repression of *dve* expression, as has been observed in the case of *lab* which is also repressed at high Wg levels (Hoppler and Bienz, 1995).

To study a potential interaction between *dve* and *lab* we analyzed *dve* expression in *lab* mutants and vice versa. *lab* is still expressed in *dve* mutants and *dve* is still expressed in *lab* mutants. Upon ubiquitous expression of *dve* in all endodermal cells using the UAS-Dve and XB30Fkh-Gal4 lines we found ectopic activation of *lab*; at early stages throughout the midgut and later most prominently in the endoderm underlying the parasegments 3, 7/8 and posterior to parasegment 9 (Fig. 7J). These embryos showed a highly aberrant midgut morphology with strong constriction defects. Remarkably, these embryos display a gut morphology which is reminiscent of the one where Dpp is ectopically expressed (Fig. 7G,J) Staehling-Hampton and Hoffmann, 1994).

## 3. Discussion

### 3.1. dve encodes a putative protein containing two homeodomains of a novel type

Our molecular analysis of the *dve* locus suggests that the *dve* transcription unit encodes two proteins; Dve, which contains two homeodomains of a novel type and the smaller Dve-s which lacks a DNA-binding motif but is identical to Dve in its N-terminal region. It is likely that the two proteins are co-expressed in the embryo. The function of Dve-s is not known. Dve-s (and Dve) contain a PEST-domain (Rechsteiner and Rogers, 1996) that might regulate the protein stability and a glutamine stretch which is known to mediate protein-protein interactions (Courey and Tjian, 1988). The analysis of *dve* mutants indicate that Dve-s is most likely not required for proventriculus development whereas Dve that carries the homeodomain function is essential. The deduced homeodomains of Dve display an unusual insertion of 10 aa between helices II and III which is not found in any other *Drosophila* homeodomain protein but shared by the putative yk117e1.5 protein of *C. elegans*. The transcription factor LFB1/HNF1 contains a 21 aa insertion between helices II and III which forms a large loop (Bürglin, 1995). Studies on the structure of its homeodomain have shown that it is similar to the one of typical homeodomains with the exception of the extra loop formed between helices II and III. This suggests that the 10 aa insertion in Dve also does not affect the structure of its homeodomains. Sequence alignment reveals that helix III of the Dve homeodomains show 64.7% identity to helix III of Orthodenticle whose DNA-binding specificity is determined by a lysine residue at position 50 (Bürglin, 1995). This residue is conserved in the Dve homeodomains and in homeodomain 2 of the yk117e1.5 protein. It is likely, that these proteins display a similar DNA-binding specificity as Orthodenticle. Furthermore Dve, the yk117e1.5 protein and the human SATB1 protein that binds selectively to the nuclear matrix-scaffold-associating DNAs (MASs/SARs) share a domain which we have named Compass domain. The function of this protein motif is, however, not known and will have to await further analysis.

### 3.2. dve is required for proventriculus development

Proventriculus development involves cell migration and adhesion processes which transform a simple epithelial tube into a folded organ structure (King, 1988; Campos-Ortega and Hartenstein, 1997). Mutant analysis and misexpression experiments have suggested previously that the genes *wg*, *hh* and *dpp* define a signalling centre in the foregut tube that coordinates these morphogenetic events which take place at the ectoderm/endoderm boundary of the foregut and the midgut (Pankratz and Hoch, 1995).

The proventriculus defect of *dve* mutants is very similar to the one that was obtained previously with a temperature-sensitive allele of wg (Pankratz and Hoch, 1995). The endodermal part of the organ is collapsed and the ectodermal oesophagus fails to invaginate into the midgut portion. This phenotype which arises also in *hh* mutants was designated earlier as 'cardiac arrest' phenotype (Pankratz and Hoch, 1995). Mutant analysis and misexpression studies show that *hh* and *dpp* activities have, if at all, only a minor influence on *dve* expression in the proventriculus. Our results are, however, consistent with the argument that *dve* is a newly identified target gene of Wg signalling that mediates the coordination of epithelial morphogenesis upon signal reception in the proventriculus. In *wg* mutants, *dve* expression in the endodermal part of the proventriculus primordium is absent and upon ectopic expression of *wg*, the *dve* expression domain becomes ectopically activated both in the ectoderm and the endoderm. The interaction of Wg and *dve* fit well with the expression patterns of the genes during gut development (Fig. 6J). *wg* is initially expressed in a domain that overlaps the ectoderm/endoderm boundary and *dve* becomes induced overlapping to *wg* in neighbouring endodermal midgut cells. Upon keyhole formation, when the *wg* domains splits, *dve* expression overlaps with the posterior *wg* domain until late stages. This overlap seems to be important since in *dve* mutants, the posterior *wg* domain is not maintained pointing towards the possibility of an autoregulatory feedback loop between *wg* and *dve*. If *wg* or *dve* are not expressed in this region, the proventriculus endoderm collapses and the invagination of the ectodermal into the endodermal tissue is defective (Fig. 1) (Pankratz and Hoch, 1995). The cause of the defect of the outer wall cells is not known. No cell death occurs in *dve* mutant proventriculi and cell counts suggest that the number of the wall cells is as in wild type. Since we possess no markers for the identity of the wall cells, we cannot exclude a misspecification of these cells in *dve* mutants. However, we favour the view that the cause for the collapsed outer wall structure are the defective shapes and adhesion properties of the endoderm cells in *dve* mutants. It has been shown previously that the cell shape changes during the folding of the proventriculus tissue require the activity of the *lethal (1) myospheroid* gene (*mys*) (Pankratz and Hoch, 1995) which encodes one of two known $\beta$ subunits of the Position Specific (PS) integrins in *Drosophila* (MacKrell et al., 1988; Leptin et al., 1989). The cell adhesion molecules of the integrin family are $\alpha\beta$ heterodimeric transmembrane receptors that mediate cell-cell and cell-extracellular matrix interactions (Hynes, 1992). The proventriculus of *mys* mutant embryos also show a collapsed outer wall structure (Pankratz and Hoch, 1995). It is therefore possible that *dve* might induce genes encoding integrins. In the posterior region of the gut *wg* is expressed at the midgut/hindgut boundary (Hoch and Pankratz, 1996) and *dve* is again activated adjacent to this domain in the endoderm. Remarkably, this expression domain of *dve* is also absent in *wg* mutants and ectopic expression of *wg* leads to an expansion of the posterior *dve* domain towards the anterior. Wg is thus reg-

ulating both the anterior and posterior *dve* expression domains in the endoderm.

### 3.3. dve is induced in the central midgut domain by Dpp and Wg signalling

The formation of the second midgut constriction has been developed into a model system for studying inductive processes in *Drosophila* (Skaer, 1993; Bienz, 1997). This endoderm structure which forms at the parasegment 7/8 boundary (Fig. 7B) is initiated by Dpp and Wg signalling activities which emanate from the overlying visceral mesoderm and induce the expression of the homeotic gene *lab* (Immerglück et al., 1990; Panganiban et al., 1990; Reuter et al., 1990). *dve* is expressed in the endoderm underlying parasegments 7 and 8 prior to the formation of the second midgut constriction and expands later towards both sides into the parasegments 6 and 9 region (Fig. 7B). In *dpp* mutants, *dve* expression in the anterior region of the central domain was reduced. Conversely, upon ectopic expression of Dpp in *abdA* mutants or when providing Dpp ubiquitously in the visceral mesoderm, we detected ectopic *dve* expression in the endoderm. A similar misexpression of *dve* could be obtained when an activated form of Tkv was ubiquitously expressed in the endoderm. These results provide strong evidence that Dpp signalling induces *dve* expression in the central midgut. Another target gene induced upon Dpp-signalling is *lab* (see Bienz, 1997, for review). In *lab* mutant *dve* expression is present and in *dve* mutants, *lab* is induced. However, when misexpressing Dve ubiquitously in the midgut endoderm we obtained almost ubiquitous misexpression of *lab* in the endoderm. These results suggest that *dve* might be part of a redundant system controlling Dpp-dependent *lab* induction in the embryo. It has been recently demonstrated, that *lab* is activated by D-Fos whose level becomes locally induced by Dpp signalling (Riese et al., 1997). In *dve* mutants, this interaction might still take place and would explain the presence of *lab* expression in the mutants. In the larval midgut, *dve* expression is repressed by *lab* and thus copper cell specification is allowed to occur (Nakagoshi et al., 1998).

However, central *dve* expression is not only *dpp*-dependent but also *wg*-dependent, as deduced from our analysis of *dve* expression in *tkv* and *shn* mutants in which both *dpp* and *wg* expression is lacking (Affolter et al., 1994; Grieder et al., 1995). In these mutants, *dve* expression was much stronger reduced than in *dpp* mutants alone. Consistent with a putative role of *wg* we found a reduction of the central *dve* expression in *wg* mutants. The overexpression studies with Wg which lead to a repression of the central *dve* domain suggests that the *dve* regulation by Wg is most likely dosage-sensitive, as has been observed in the case of *lab* regulation (Hoppler and Bienz, 1995). The analysis of *abdA* mutants indicates, however, that Dpp signalling is critical for central *dve* expression and can induce *dve* without *wg* being expressed. The deduced roles of Dpp and Wg signalling in regulating central *dve* expression are consistent with *dpp* and *wg* being expressed in a region overlying the *dve* expression domain in the endoderm (Fig. 7B).

## 4. Experimental procedures

### 4.1. Fly stocks and ectopic expression studies

The *dve* alleles $dve^{P1738}$, $dve^{P1829}$, $dve^{P5084}$ and $dve^{P6432}$ correspond to the P element insertion lines l(2)01738, l(2)01829, l(2)05084 and l(2)06432 (Karpen and Spradling, 1992). The X-ray induced deficiency Df(2R)58–5 spanning the *dve* transcription unit was a gift of C. Klämbt (Münster). For epistatic analysis we used the following stocks: OregonR, $wg^{IL114}$, $wg^{CX4}$, $arm^{XK}$, $dpp^{S4}$, $dpp^{BC86}$ $abdA^{M1}$, $Ubx^{MX12}$, $shn^{TD25}$, $lab^{vd1}$, $hh^{IJ35}$ (provided by the Bloomington and Tübingen stock centres). The flies were maintained and embryo collections were made according to standard procedures.

For the GAL4/UAS experiments we used the Gal4 driver lines twist-Gal4 (a gift of M. Frasch), Fkh XB30-Gal4 (R. Pflanz and M. Hoch, unpublished data) Fkh 14–3-Gal4 (S. Stein and M. Hoch, unpublished data) and the UAS lines UAS-Wg (a gift of S. Cohen), $UASTkv^{QD}$ (a gift of K. Basler), UAS-Dpp (a gift of S. Cohen). Mutants were scored using blue balancers. To generate a UAS-Dve transgene, we cloned a 5.0 kb XhoI/Xba fragment which includes the open reading frame of the 5.0 kb *dve* transcript (including intron 3) into the XhoI/XbaI site of the pUAST vector (Brand and Perrimon, 1993) The transgene was introduced into the germline of $w$ $sn^{w}$ homozygous mutant embryos by P element-mediated germline transformation (Rubin and Spradling, 1982). Several independent transgenic fly strains bearing pUAST-Dve transgenes on different chromosomes were obtained. Embryos were collected at 29°C and analyzed by RNA in situ hybridization or antibody stainings.

### 4.2. Molecular characterization of the dve locus

Preparation of DNA, Southern and Northern blot analysis were carried out according to standard procedures (Sambrook et al., 1989). Genomic DNA flanking the P insertion of the *dve* alleles was obtained by plasmid rescue experiments (Karpen and Spradling, 1992). Six phages encompassing the *dve* transcription unit were isolated from a genomic *Drosophila* CantonS DNA library (Stratagene) and partially sequenced. The mapping and characterization of the phages was confirmed on the sequence level by the 59 kb P1-clone DS09121 from the Berkeley *Drosophila* Genome Project. Twelve cDNA clones were obtained from a 8–24 h plasmid cDNA library (a gift of N. Brown), a 4–12 h plasmid cDNA library (Clontech) and a 8–24 h phage cDNA library (λ-Zap, Stratagene). Sequences of genomic DNA and cDNA were determined by the dideoxynucleotid method (Sambrook et al., 1989) using automated sequen-

cing. Sequence similarity searches were performed at NCBI using blastp and blastn programs.

### 4.3. In situ hybridization and immunocytochemistry

RNA in situ hybridization of whole mount embryos was carried out with digoxigenin-labelled and flourescein-labelled RNA probes (Tautz and Pfeifle, 1989) with modifications for the detection reaction. In our experiments we used RNA probes from *wg*, *dpp*, and *hh* (kind gifts of S. Cohen), *otp* (kind gift of U. Walldorf). For whole-mount immunostainings we applied the VECTASTAIN Elite ABC-peroxidase system (Vector Laboratories) with DAB as substrate. As markers for gut development we used anti-Fkh antibody (kind gift of P. Carrera), the monoclonal antibody anti-Fas III (gift of D. Brower), a monoclonal anti-Wg antibody (gift of S. Cohen) and a polyclonal anti-Lab serum (gift of M. Affolter).

### 4.4. Phenotypic and molecular analysis of the dve alleles

In a feeding assay, we used yeast dyed with Carmine red (Sigma) to visualize transport of food along the alimentary canal of mutant larvae (Pankratz and Hoch, 1995).

Differential analysis of *dve* transcripts was investigated by RT-PCR applying the Titan-One-tube system from Boehringer. One reaction was performed with 100 ng of poly (A + ) RNA of wild type or *dve* mutant larvae that were scored using the feeding assay. We used one upstream oligonucleotide U1 (5′-CGACGCGATCAGCTCCC-GATTTCATGG-3′) and two downstream oligonucleotides D1 (5′-GCTCCTCCCGATCCCGATCCCTCTC-3′) and D2 (5′-TCCCGCTCCTCGTACTTGGGCACAAAAG-3′). The corresponding 3525 bp (U1–D2) and 1055 bp (U1–D1) RT-PCR products were confirmed by Southern analysis using a 820 bp NotI fragment from the 4.85 kb *dve* cDNA as a probe.

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