Cross regulation of *decapentaplegic* and *Ultrabithorax* transcription in the embryonic visceral mesoderm of *Drosophila*

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**Summary**

The *Drosophila decapentaplegic* gene (*dpp*) encodes a TGF-β family member involved in signal transduction during embryonic midgut formation. The shortvein (*shv*) class of cis-regulatory *dpp* mutants disrupt expression in parasegments 4 and 7 (ps4 and ps7) of the embryonic visceral mesoderm (VM) surrounding the gut and cause abnormalities in gut morphogenesis. We demonstrate that cis-regulatory elements directing expression in ps4 and ps7 are separable and identify DNA fragments that generate ps4 and ps7 expression patterns using reporter gene constructs. *dpp* reporter gene expression in both ps4 and ps7 is autoregulated as it requires endogenous *dpp* activity. Reporter gene ps7 expression requires the wild-type action of *Ultra-bithorax (Ubx)*, and *abdominal-A*. Furthermore, the expression of certain *Ubx* reporter genes is coincident with *dpp* in the VM. Both the mis-expression of *Ubx* reporter genes in the developing gastric caecae at ps4 and its normal expression in ps7 are dependent upon endogenous *dpp* activity. We conclude that *dpp* both responds to and regulates *Ubx* in ps7 of the visceral mesoderm and that *Ubx* autoregulation within this tissue may be indirect as it requires more components than have previously been thought.

**Key words:** *Drosophila, decapentaplegic, Ultrabithorax, visceral mesoderm, cis-regulation, TGF-β superfamily*

**Introduction**

Interacellular communication plays a major role in elaborating developmental decisions. The *decapentaplegic* (*dpp*) gene coordinates such cell-cell signalling in a number of patterning events during *Drosophila* development. DPP is a member of the transforming growth factor-β (TGF-β) family of secreted signalling proteins (Padgett et al., 1987). Its expression pattern throughout development is temporally and spatially complex, and is regulated by a variety of spatially restricted transcription factors. *dpp*’s earliest spatial restriction is to the dorsal 40% of the early embryo (St. Johnston and Gelbart, 1987). This restriction is essential to its role in establishing dorsal ectoderm (Irish and Gelbart, 1987; Ray et al., 1991) and occurs through negative regulation in ventral regions by the *dorsal* protein, a transcription factor with homology to the mammalian transcription factor NF-κB (Steward, 1987; Kieran et al., 1990; Ghosh et al., 1990). The expression of *dpp* at or near the anterior/posterior compartment boundary in imaginal disks is necessary for the proximal-distal growth of all adult appendages (Spencer et al., 1982) in a pathway negatively regulated by the homeodomain gene *engrailed* (Raftery et al., 1991).

In the development of the embryonic midgut, *dpp* is involved in passing determinative information between tissue layers (Immerglück et al., 1990; Reuter et al., 1990; Panganiban et al., 1990). The embryonic midgut comprises endoderm surrounded by a thin layer of mesoderm called the splanchnopleura or visceral mesoderm (VM). During early development, the midgut is an open tube, and the VM appears as two broad bands running laterally along this tube. As embryogenesis proceeds, this tube closes and the VM becomes a thin sheath enclosing the underlying endoderm. At this point, cell shape changes produce three constrictions that divide the midgut into compartments (Campos-Ortega and Hartenstein, 1985; Reuter and Scott, 1990). Two areas of the VM show strong *dpp* expression (St. Johnston and Gelbart, 1987). These sites roughly underlie parasegments 4 and 7 (ps4, ps7) of the ectoderm, at the sites in the anterior midgut where the gastric caecae evaginate and the secondary midgut constriction forms, respectively. (Although these patches of expression may not actually be parasegmental in nature, we shall use parasegmental markers to simplify the description of their positions.) Rearrangement breakpoints in the proximal 5′ nonprotein-coding region of the *dpp* gene disrupt this VM expression and cause abnormalities in gut morphogenesis (Immerglück...
et al., 1990; Reuter et al., 1990; Panganiban et al., 1990). Specifically, the gastric caeca fail to evaginate and the secondary midgut constriction does not form.

The products of many homeotic genes are expressed in the developing midgut. Sex combs reduced (Scr) is normally expressed in the VM directly posterior to the ps4 domain of dpp. Loss of Scr expression results in a failure to form gastric caeca (Reuter and Scott, 1990). In addition, in dpp genotypes lacking dpp expression in ps4, the Scr domain expands into the dpp ps4 domain. Thus dpp represses Scr expression in ps4 (Panganiban et al., 1990).

In ps7 of the VM, dpp expression is coincident with that of the Ubx gene (White and Wilcox, 1985; Bienz et al., 1988; Immerglück et al., 1990). Genotypes that result in loss-of-function for either Ubx or dpp in ps7 result in indistinguishable mutant ps7 midgut phenotypes (Immerglück et al., 1990; Panganiban et al., 1990). In Ubx loss-of-function alleles, dpp product does not accumulate to wild-type levels in ps7 (Immerglück et al., 1990; Reuter et al., 1990). Thus dpp’s expression in ps7 is dependent on Ubx although a direct interaction has not been demonstrated. However, conflicting results have been presented for dpp’s effect on Ubx protein levels. Panganiban et al. (1990) found a reduction in the amount of Ubx protein in a shv mutant background, while Immerglück et al. (1990) reported that the level of Ubx protein in ps7 is unaffected in a shv mutant background.

The homeotic gene, labial (lab), is expressed in the endoderm underlying ps7 of the VM. In embryos homozygous for Ubx loss-of-function mutations or shv mutations, lab is not expressed in the endoderm, indicating that dpp and Ubx are required for lab expression (Immerglück et al., 1990; Reuter et al., 1990). Immunohistochemical studies demonstrate that the dpp protein is transferred from its site of synthesis in the VM to putative target cells of the underlying endoderm (Panganiban et al., 1990). Expansion of the Ubx domain either by its derepression in loss-of-function abdominal-A (abd-A) or Polycomb (Pc) backgrounds, or by its ectopic expression in a heat shock Ubx construct, shows that Ubx expression is sufficient to expand both the dpp and lab domains in the midgut (Immerglück et al., 1990; Reuter et al., 1990). Thus in ps7, the passage of a dpp signal integrates gene expression across germ layers.

The activities of dpp in the midgut represent a system where the action of a signal transduction pathway causes readily identifiable morphogenetic change: the cell shape changes resulting in either evagination or constriction of tissues. We are interested in understanding how transcription factors and signal transduction molecules collaborate to effect morphogenesis. We ultimately hope to determine if the interaction between dpp and Ubx represents a direct regulation of dpp transcription by Ubx, and what other factors are involved in this regulation. As a first step in such an analysis, we have investigated the regulation of dpp’s transcription in the VM.

In this report we localize the cis-regulatory sites in dpp controlling its expression in ps4 and ps7 in the VM and demonstrate for the first time that ps4 and ps7 regulatory elements map to separate regions within the 5’ noncoding region. We identify DNA fragments that generate bona fide ps4 and ps7 dpp expression patterns in reporter gene constructs. Using these reporter genes we demonstrate autoregulation of dpp expression in both ps4 and ps7. Furthermore, we provide evidence that the previously identified autoregulation of Ubx in ps7 requires dpp expression and may be an indirect and complex feed-back pathway.

**MATERIALS AND METHODS**

**Plasmid constructions**

P20: A 20 kb Sall fragment (dpp coordinates 71.2-92) derived from dp cn cl bw was subcloned from a cosmid into Carnegie 20 (Rubin and Spradling, 1983).

P15: A 14.4 kb Xhol-Sall fragment (dpp coordinates 77.5-92) derived from dp cn cl bw was subcloned from a cosmid into Carnegie 20.

RD1: A 6.3 kb BamHI fragment (dpp coordinates 75.1-81.4) was cloned from P20 into the BamHI site of Casper-β-galactosidase-AUG (Thummel et al., 1988).

RD2: An 8.9 kb EcoRI fragment (dpp coordinates 67-75.9) was subcloned from the 68R plasmid derived from Canton-S into pHSF6 (provided by Kevin Jones). The resultant plasmid was digested with NorI to release a fragment from NorI sites in the plasmid flanking the Drosophila DNA. This fragment was subcloned into the NorI site of HZ500PL (Hiromi and Gehring, 1987).

**Germ-line transformation**

P-element transformation was carried out using standard protocols (Spradling, 1986). The above constructs (at 400 µg/ml) and pnt25.7/w DNA (at 100 µg/ml; Karess and Rubin, 1984) were coinjected into cn; ry506 or y w embryos. Each insertion was mapped to a chromosome and made homozygous. In most cases, additional inserts were generated by mobilization of a primary transformant using the Δ2-3 (99B) genomic source of P transposase (Robertson et al., 1988).

**Drosophila strains and constructions**

dpp alleles are described elsewhere (Segal and Gelbart, 1985; St. Johnston et al., 1990). The alleles of homeotic mutations were as follows: Ubx9.22, Ubx6.28 (Kerridge and Morata, 1982); Ubx109 (Lewis, 1978) and abd-Ams-2 (Sanchez-Herrero et al., 1985). The Ubx reporter gene, 15-1 wt/△UBx, contains the region from -3.1 to +900 of the Ubx gene, fused to a nuclear β-galactosidase gene and was provided by Philip Beachy.

**Drosophila embryo analysis**

The phenotypes of mutant embryos were inferred from examining large numbers of progeny and looking for consistent defects in expression observable in roughly 1/4 (the expected frequency of mutant homozygotes or double heterozygotes) of the embryos of the appropriate stages. With later embryos, dpp or Ubx derived gut abnormalities could be used to confirm genotypes. Embryos were staged according to Campos-Ortega and Hartenstein (1985). The effect of homozygous dppshv mutations on dpp reporter gene constructs was assessed in dppshv mutant stocks balanced with a CyO chromosome bearing a wingless-β-galactosidase reporter gene (provided by Norbert Perrimon), and homozygous for insertions of either the RD1 or RD2 construct. Similarly marked balanced strains were used to examine RD1 in Ubx and abd-A mutant backgrounds. A TM3 balancer, bearing a fushi tarazu-β-galactosidase reporter gene (Hiromi and Gehring, 1987) was used to distinguish critical genotypes. The P20 and P15 rescue constructs were assayed in dpp null backgrounds. Embryos homozygous for a dpp null allele dapc1a and homozygous for P20 or P15 were generated from progeny of a balanced stock of the following geno-
type: dpp$^{1465}$ CyO,Dp dpp$^{+}$: P=+/P=−. One quarter of the embryos from this strain are null for the endogenous dpp gene and homozygous for either the P20 or P15 rescue construct. For Ubx expression studies, segregants were identified by simultaneously monitoring expression of Scr in the VM. In the absence of dpp, the Scr domain is increased in size. For lab expression, mutant phenotypes were inferred on the basis of expected Mendelian ratios.

**Histochanical β-galactosidase staining**

Embryos were dechorionated by standard methods and fixed in 4% formaldehyde in PBS (340 mM NaCl, 6.6 mM KCl, 3.7 mM KH$_2$PO$_4$, pH 7):n-heptane, 1:1. Embryos were washed in PBS and stained for β-galactosidase activity according to Blackman et al. (1991). Embryos were mounted in PBS or glycerol and photographed with Nomarski optics.

**Antibody staining of embryos**

Rabbit polyclonal anti-β-galactosidase antibody was provided by Robert Holmgren. Monoclonal antibody recognizing Ubx, FP3.38 (White and Wilcox, 1984) was provided by Danny Brower, and anti-Scr monoclonal antibody was provided by James Mahaffey. Embryos were dechorionated by standard methods and fixed according to Raftery et al. (1991). Endogenous peroxidases were inactivated by a 15 minute treatment in a 9:1 mixture of MeOH and H$_2$O$_2$ (30%). Blocking was accomplished with two 30 minute washes in PBS with 0.1% Triton X-100 (PBT) and 10% normal goat serum (NGS). Embryos were incubated overnight at 4°C with antibodies diluted in PBT and 5% NGS (1:1000 for FP3.38, 1:2000 for Scr, 1:1000 for anti-β-galactosidase) and washed in PBT. They were then incubated for 2 hours at room temperature with secondary antibodies directly conjugated to horseradish peroxidase and diluted 1:2000 in PBT, washed in PBT and stained with 3,3′-diaminobenzidine tetrahydrochloride for up to 30 minutes. Embryos were mounted in methyl salicylate and photographed with Nomarski optics.

**Whole-mount in situ hybridizations**

Digoxigenin whole-mount in situ hybridizations were done according to the protocol of Tautz and Pfeifle (1989). Digoxigenin-labeled fragments were prepared as described in Ray et al. (1991). Plasmids used as probes were pBEl1, a 4.5 kb dpp cDNA in pNB40 (St. Johnston et al., 1990) and a 1.7 kb EcoRI fragment of a labial cDNA inserted into Bluescript (provided by Thomas Kaufman). Embryos were mounted in glycerol and photographed using bright-field or Nomarski optics.

**RESULTS**

**Mutations in the shortvein region of dpp selectively remove aspects of embryonic visceral mesoderm expression of dpp**

The shortvein (shv) region of dpp comprises the area 5′ to the dpp coding exons (Fig. 1). This area contains all but one of the promoters that drive the transcripts encoding the single dpp polypeptide (St. Johnston et al., 1990). Most dpp$^{shv}$ homozygous or trans-heterozygous mutant combinations are larval lethals (Segal and Gelbart, 1985; Hursh and Ray, unpublished observations). The majority of dpp$^{shv}$ mutants are chromosomal rearrangements that sever portions of the 5′ region from the transcription units they control. These mutations effectively act as deletions of all material 5′ to their shv breakpoints. Mutants that delete most of the shv region from the dpp gene disrupt dpp expression in the visceral mesoderm and exhibit gut defects (Immerglück et al., 1990; Panganiban et al., 1990). Previous reports examined only a few of the available shv mutations. We wished to analyse the entire region in more detail to localize potential cis-regulatory information.

Transcript localization by whole-mount digoxigenin in situ hybridization was carried out on both homozygous and trans-heterozygous combinations of the dpp$^{shv}$ alleles indicated in Fig. 1 (for ease of presentation, shv mutations will be indicated only by their unique superscripts, i.e., dpp$^{s4}$ will be called s4). Transcripts are detected in the clypeolabrum, pharynx, esophagus and sites of the evaginating gastric caeca (ps4) and the secondary midgut constriction (ps7) of wild-type animals (Fig. 2A,B). Some preparations also show expression at the site of the future third midgut

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**Fig. 1. Schematic diagram of the dpp gene.** The coordinates of the gene (St. Johnston et al., 1990) are indicated across the top. The location of the exons for the dpp transcripts are immediately below. Black boxes represent protein coding exons and stippling indicates the 3′ untranslated region. White boxes represent five alternative 5′ noncoding exons that are spliced into the first coding exon at exactly the same site. The 5′ ends of the 2 most proximal noncoding exons have been determined by S1 analysis (St. Johnston, 1988); the others are inferred by the positions of unique cDNAs. The shv region begins at molecular coordinate 83, as defined by the s13 breakpoint. Its 5′ limit is unclear. The extents of the RD1 and RD2 reporter gene constructs are indicated by black bars, and the extents of the P20 and P15 rescue constructs by stippled bars. Mapped positions of shv breakpoints (St. Johnston et al., 1990) are at bottom. With the exception of s6, all the shv mutations are chromosomal rearrangements. Black lines represent extent of DNA removed from the dpp transcription unit by a given mutation, with cross-hatched boxes representing the uncertainty of location of the endpoint of each mutation.
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constriction (Fig. 2I). Breakpoints that remove DNA sequences distal to molecular coordinate 82.7 (s13, s4, s18, s25, see Fig. 1) eliminate all aspects of internal dpp expression (pharynx, esophagus, ps4, ps7; Fig. 2C) without alteration of the complex ectodermal expression pattern. Late embryos fail to evaginate gastric caecae or form secondary midgut constrictions and the primary midgut constriction is shifted posteriorly (Fig. 2D, also see Panganiban et al.,

Fig. 2. Expression of dpp transcripts in wild-type and homozygous shv mutant embryos detected by whole-mount in situ hybridization. In this and in subsequent figures, anterior is to the left. A,C,E,G,I are lateral views; B,D,F,H,J are horizontal views. In later embryos, midgut constrictions are indicated by numbers. Dark staining on the periphery of embryos represents the ectodermal expression of dpp, that is unaltered in these genotypes. (A) Stage 13, wild-type embryo. Expression is seen in the clypeolabrum (c), pharynx (p), esophagus (e), and ps4 (arrow) and ps7 (arrowhead) of the VM. (B) Stage 16 wild-type embryo. Transcripts are detected at the sites of the evaginating gastric caecae (arrows) and surrounding the secondary compartment of the midgut (arrowheads). (C) Stage 13 of an s4 homozygote. Clypeolabrum expression is detected (c), but all other internal expression of dpp is absent. (D) Stage 16, of an s4 homozygote. No dpp expression is observed. The gastric caecae are absent and the midgut is missing a compartment. (E) Stage 13 of an s22 homozygote. Staining in the clypeolabrum (c) and at the ps7 position of the VM (arrowhead) is observed. (F) Stage 16 of an s22 homozygote. Expression in the VM at the anterior portion of the midgut is absent, as are the gastric caecae. Expression in the VM surrounding the second gut compartment is normal (arrowheads). (G) Late stage 13 of an s6 homozygote. Staining is observed in the clypeolabrum (c), pharynx (p), and ps4 region of the VM (arrow), although this latter staining appears reduced as compared to wild type. No expression is seen at ps7.

Esophageal expression can be seen in dark preparations of this genotype. (H) Late stage 16 s6 homozygote. Transcript is undetectable at this stage, but gastric caecal evaginations are observed. Note abnormal gut morphology. (I) Stage 13 s11 homozygote. Clypeolabrum expression is observed (c). No expression is detected in the pharynx or esophagus. Expression is visible in the ps4 region, albeit at a lower level than in wild type (compare ps4 and ps7 expression in (A) and in (I)). ps7 VM expression is normal (arrowhead). An additional site of expression posterior to ps7 is observed in this preparation (white arrow). This is a normal site of dpp expression (St. Johnston and Gelbart, 1987), observed only in darkly stained preparations. (J) Stage 16 of an s11 homozygote. Faint expression is observed at the evaginating gastric caecae (arrows).
duce gastric caeca. The results obtained with dpp expression in the VM at ps7 (Fig. 2E,F). The pharyngeal, esophageal and ps4 VM expression of dpp are still absent, indicating that spatial cis-regulatory information controlling these aspects of dpp expression are located distal to the s22 breakpoint. In agreement with these expression data, s22 mutant embryos have normal midgut constrictions, but lack gastric caeca (Fig. 2F). This result is supported by examination of one of only two internal deficiencies that exist in the shv region, s6 (Fig. 1). In this mutation, ps7 VM staining is absent, and the embryos are missing the secondary constriction (Fig. 2G,H). Staining in the ps4 region of the VM appears to be reduced when compared to wild type (Fig. 2G), and is undetectable later in development (Fig. 2H), but these embryos nonetheless produce gastric caeca. The results obtained with s6 also suggest that the ps7 VM dpp expression originates between coordinates 76 and 72. Finally, in embryos homozygous for s11 whose proximal breakpoint is approximately 74 (Fig. 2I), expression in ps7 is normal while expression in ps4 is reduced but detectable (Fig. 2I). The midgut constrictions of s11 homozygotes are normal (Fig. 2J). However, the gastric caeca, although present, may be somewhat reduced in length. The dpp staining in the pharynx and esophagus is also missing in this genotype, placing their regulatory elements distal to the s11 breakpoint.

In summary, these analyses allow us to crudely order regulatory regions within the shv region. The pattern of expression in ps7 suggests that it is controlled from the most proximal position, with expression in ps4 closely apposed but distal to it. The expression of dpp in the pharynx and esophagus must also be directed by the shv region with control elements located more distally than those directing ps4 expression.

Sequences in the shv region are both necessary and sufficient to direct dpp's VM expression

To further define the sequence requirements for dpp gene expression in the VM, we compared two rescue constructs, P20 and P15 (Fig. 1). These two constructs differ only in their distal endpoints and contain the same set of dpp promoters. Both constructs extend to the end of the coding region (Hin region) but contain none of dpp’s extensive 3’ cis regulatory unit (disk region). P20 extends to 71.2 on the molecular map, a position further distal than the s11 breakpoint. Given the result described above with s11, we predicted that P20 would contain the gastric caeca and ps7 response elements, and indeed, this construct rescues shv-associated larval lethality (data not shown.) In contrast, P15 extends distally only to 77.6 on the molecular map. The P15 construct expresses functional dpp as evidenced by its ability to rescue the dominant D/V patterning defects of dpp null mutations (data not shown). We compared the ability of these two constructs to drive dpp expression in the VM in homozygous dpp null backgrounds. We were unable to examine dpp transcript directly, as the dpp loss-of-function alleles we used were not RNA nulls. We instead examined expression of Scr, Ubx and lab. Lack of dpp expression in ps4 causes an expansion of the Scr domain (Panganiban et al., 1990) and in ps7 causes an elimination of lab expression (Immerglück et al., 1990; Panganiban et al., 1990). In contrast, in P15 backgrounds, approximately 1/4 of the embryos examined displayed Scr domain expansion (Fig. 3B). In these embryos, which were inferred to express dpp solely from the P15 rescue construct, Ubx expression in ps7 was reduced (Fig. 3B). Gut defects were observed in older embryos. When lab expression was monitored using digoxigenin in situ hybridization, approximately 1/4 of the appropriate stage embryos failed to express lab in the gut endoderm (data not shown).

We conclude that the sequences contained in P15 are insufficient for correct dpp expression in ps4 and ps7 of the VM. That is consistent with the results obtained with shv mutants and targets the region between 77.6 and 71.2 as critical in dpp’s VM expression. In addition, the ability of P20 to provide both spatially correct expression and rescue
ps4 to ps7 expression is that observed for wild-type dpp expression. (E,F) Stage 15 and 16 embryos, respectively. Expression in ps4 and ps7 follows the normal dpp pattern (compare to Fig. 2A,B). In addition, expression is also observed in the developing esophagus (arrow) and, posterior to ps7, at the site of the tertiary midgut constriction (arrowheads).

do larval lethality indicates that no further sequences from the dpp gene are required for VM function and gut morphogenesis.

**Location of cis elements directing dpp VM expression in the shv region**

Our conclusions are supported and supplemented by two β-galactosidase reporter constructs, RD1 and RD2 (Fig. 1). RD1 uses the resident dpp promoter(s) to drive β-galactosidase expression, while RD2 places shv region sequences upstream of a minimal hsp70 promoter. The lacZ expression patterns produced by these constructs are shown in Fig. 4. RD1, whose coordinates are 75.1-81.4, is expressed primarily in ps7 (Fig. 4A-C). Some expression is detectable in ps4, indicating that a ps4 cis-regulatory element resides within it. However, this expression is at a much lower level than normal when compared to the ratio of expression in ps4 to ps7 observed in wild-type (Fig. 2A,B). This suggests that other elements required for wild-type ps4 pattern are absent in RD1. RD2 provides normal levels of expression in ps4 relative to ps7, suggesting that additional elements driving gastric caecae expression are located within RD2 but not within RD1 (Fig. 4D-F). In both constructs, the spatial restriction of expression is indistinguishable from dpp transcript accumulation. Consistent with our genetic analysis, in RD2, β-galactosidase expression is detectable in the embryonic esophagus (arrow, Fig. 4E,F). We also observe faint expression in the area where the tertiary midgut constriction will form (arrowheads, Fig. 4E,F). These are both sites of normal dpp expression (St. Johnston and Gelbart, 1987), although no defects have been ascribed to loss of dpp activity in these areas. Note that, overall, RD2 expresses more strongly than RD1.

dpp VM reporter gene constructs respond to BX-C gene products

The response of dpp expression in the VM to mutations in genes of the bithorax complex (BX-C) has been defined by the use of mutants and gene-specific probes (Immerglück et al., 1990; Reuter et al., 1990; Panganiban et al., 1990). These results indicate that Ubx is a positive activator of dpp’s VM expression, and that in embryos homozygous for abd-A mutations the domain of dpp expression expands posteriorly in a manner identical to that observed for Ubx itself (Bienz and Tremml, 1988). We wished to know if the cis-regulatory elements contained in RD1 and RD2 responded to homeotic genes in the same way. Fig. 5 shows the response of RD1 and RD2 to a loss-of-function allele of Ubx. (The allele shown is Ubx9,22. The result with Ubx6,28 is identical.) In the absence of Ubx gene function, the ps7-specific lacZ expression of RD1 and RD2 is abolished or reduced, respectively (Fig. 5B,F). (Low levels of expression from RD1 could be present but below detectable levels, due to its weaker wild-type expression.) Lack of abd-A activity yields ectopic expression of β-galactosidase in the VM surrounding the midgut (Fig. 5C,G). In Ubx109, a deficiency removing both the Ubx and abd-A genes (Karch et al., 1985), strong expression is observed in the most posterior portion of the midgut from both RD1 and RD2 (Fig. 5D,H). A similar result has been observed for Ubx reporter genes in this genetic background (Bienz and Tremml, 1988). They demonstrate that this ectopic expression is caused by the Abd-B gene in the absence of functional abd-A gene product. Mutations that remove the entire BX-C do not display this ectopic expression. Our result indicates that dpp, like Ubx, responds to this activity of Abd-B.
Surprisingly, in Ubx109, the RD2 construct expresses ectopically in the remainder of the posterior midgut in a pattern like that observed for homozygous abd-A mutant embryos, albeit at a lower level (arrowheads, Fig. 5H). As this mutation deletes both the Ubx and abd-A genes, this ectopic expression cannot be mediated by de-repression of Ubx, and must therefore suggest an independent activity of abd-A on dpp. ps4 expression is unaffected in all these mutant backgrounds.

**Autoregulation of dpp in the VM**

TGF-β1 has been reported to positively regulate its own expression in both normal and transformed cell lines (Van Obberghen-Schilling et al., 1988). We examined the response of RD1 and RD2 in shv mutant backgrounds to ask if dpp also displays positive autoregulation. We used three alleles, each with a different VM expression profile. s4 removes all dpp expression in the visceral mesoderm, s6 removes only expression in ps7, and s22 removes only expression in ps4 (Fig. 6). The response of RD1 in s4 homozygous mutant embryos is shown in Fig. 6A,B, visualized by anti-β galactosidase immunohistochemistry. Reporter gene expression in ps7 is reduced relative to controls. We also examined s6 and observed the same result (data not shown). In RD2, expression in both ps4 and ps7 was monitored. In s4, expression of RD2 is absent in ps4 and reduced in ps7 (Fig. 6C,D). RD2 reporter constructs in s6 give normal expression in ps4, but reduced expression relative to the control in ps7 (Fig. 6E,F). This expression pattern follows the distribution of dpp in this mutant. In s22, normal levels of expression are seen in ps7 but none is detectable in ps4, again paralleling the expression of dpp in embryos of this genotype. Therefore, normal RD1 and RD2 reporter gene expression levels require endogenous dpp expression, demonstrating that a positive autoregulatory loop maintains dpp’s expression in both ps4 and ps7 of the VM.

**Ubx reporter gene expression in the VM is coincident with and requires dpp**

dpp’s positive autoregulation must be mediated by one or more transcription factors. In ps7, Ubx is an obvious candidate gene, given its coincident expression and its ability to affect dpp expression (Fig. 5; also see Immerglück et al., 1990; Reuter et al., 1990). In addition, Ubx has been reported to be required for the maintenance of its own transcription in an ‘autocatalytic’ loop (Bienz and Tremml, 1988; Müller et al., 1989). However, as discussed in the Introduction, the effect of dpp on Ubx protein levels was unclear. Our results, as shown in Fig. 3, support those of Panganiban et al. (1990) and are in contrast to those reported by Immerglück et al. (1990). Thus we support the
contention that dpp affects the level of Ubx protein. However, these experiments do not determine the mechanism of this effect. We investigated whether dpp could influence Ubx transcription by examining the expression of a Ubx-lacZ reporter gene. In wild-type backgrounds Ubx β-galactosidase expression is seen in the VM surrounding the midgut at ps7, but to our surprise it is also observed in the evaginating gastric caecae at ps4 (Fig. 7A,B). The VM at the site of the gastric caecae is not a part of Ubx’s in vivo expression (Akam and Martinez-Arias, 1985; White and Wilcox, 1985; Tremml and Bienz, 1989; and our unpublished observations). However, examination of the literature (Müller et al., 1989; Irvine et al., 1991), as well as our own observations of two additional reporter gene constructs (data not shown), suggest that Ubx-reporter gene constructs containing only the region 3–5 kb upstream of the start of Ubx transcription fused to lacZ are indeed expressed in both the normal ps7 location and in the ectopic location in ps4, although gastric caecal staining in these constructs has not been noted as such. Reporter gene constructs containing larger sequences of the Ubx gene (35 kb) do not exhibit this ps4 expression (Irvine et al., 1991), suggesting that the removal of upstream DNA derepresses Ubx expression in ps4. This expression precisely matches the expression of dpp in ps4 (compare Fig. 4 and Fig. 7A,B).

This Ubx-directed β-galactosidase expression in both ps4 and ps7 is dependent upon dpp. In s4 embryos, which do not express dpp in the VM, no Ubx reporter gene activity is seen (Fig. 7C). In s6, where dpp is present in ps4 but absent in ps7, the reporter gene is expressed at wild-type levels in ps4, but expression is severely reduced in ps7 (Fig. 7D). The reciprocal expression pattern is produced by s22; expression is lost in ps4, but is normal in ps7. We do not feel that this is solely due to the absence of gastric caecal tissue, as Fig. 7A shows significant β-galactosidase expression prior to any substantial caecal evagination. In conclusion, the expression of these truncated Ubx-reporter genes is coincident with dpp in the VM, and like the dpp reporter genes RD1 and RD2, is dependent on dpp gene activity to achieve normal expression levels.

DISCUSSION

Properties of cis-regulatory elements directing VM in the shv region

We have localized cis-regulatory elements driving dpp expression in the VM surrounding the embryonic midgut by mutant breakpoints, rescue constructs, and β-galactosidase reporter genes. The properties of the cis-regulatory elements directing dpp’s expression in ps4 differ from those directing expression in ps7. Expression of dpp in ps4 is controlled...
by three or more separable elements within the shv region. First, the RD1 reporter gene construct is capable of directing a low level of ps4 β-galactosidase expression, and thus must contain ps4 regulatory information. This region in isolation appears to be insufficient to produce gastric caecae because in the s22 mutation, which retains this region, we see no detectable expression of dpp transcript in ps4, a failure to produce gastric caecae and an expansion of the domain of Scr (data not shown). The slightly more distal s11 mutation restores dpp RNA levels in ps4 to detectable but reduced levels, produces gastric caecae and represses Scr in ps4. It must therefore identify a second element required for correct dpp expression in this region, residing between the proximal end of s11 and the proximal end of s22. However, the s6 deficiency removes this element yet produces an RNA expression pattern similar to s11, produces gastric caecae and blocks Scr expression in ps4. We therefore conclude that at least one more element must reside distal to the s11 breakpoint that is contributing to gastric caecal expression in s6. The RD2 reporter construct that produces wild-type levels of expression in ps4 extends over the entire domain defined as critical by the s22, s11 and s6 breakpoints. The ability of s11 and s6 to produce spatially correct but reduced levels of expression from mutually exclusive areas of the shv region suggests that these multiple elements function in the same way. We hypothesize that wild-type expression in ps4 is achieved through additive effects of multiple elements residing in the shv region.

In contrast to the situation in ps4, expression in ps7 behaves as if there is a small regulatory region that is both necessary and sufficient to direct dpp expression in ps7. Mutant analysis suggests that DNA from molecular coordinate 72.5 (the distal-most possible position of the s22 breakpoint) to 76.6 (the proximal-most possible position of the s7 breakpoint) is necessary to affect both expression of dpp transcript in ps7 and the formation of the secondary midgut constriction. No further defects arise by removal of more proximal DNA (s12-s4). The s6 deletion removes this critical area and lacks ps7 expression, further supporting the notion of a discrete region controlling ps7, as does the inability of the P15 rescue construct to direct dpp expression in ps7. The RD1 and RD2 reporter gene constructs both direct correct spatial patterning in ps7 and their overlap includes a portion of this critical region. The most parsimonious explanation of these data is that critical ps7 elements are confined to the 800 bp between these two constructs: 75.1 and 75.9 on the molecular map. In summary, all these data are consistent with a discrete location immediately proximal to the s22 breakpoint that is necessary to direct dpp’s expression in ps7.
dpp requires multiple factors to achieve correct gene expression in the VM

The transcription of β-galactosidase in dpp reporter constructs is modulated by homeotic genes of the BX-C (Fig. 5) in a manner identical to dpp transcript (Immerglück et al., 1990) or protein (Reuter et al., 1990). Thus both RD1 and RD2 contain sufficient homeotic gene-responsive elements to produce the expected patterns of dpp expression in ps7 of the VM. We have scanned the sequence of dpp (R. W. Padgett, M. de Cuevas, S. Findley and W. Gelbart, unpublished data) covering most of our reporter genes for sites that might be predicted to bind Ubx, using a consensus binding site and related sites as defined in Ekker et al. (1991). Within the dpp sequence extending from 71.8 on the molecular map to the end of the coding exons (Fig. 1), 31 sites exist that would be predicted to bind Ubx. Over a third of these sites are clustered in the interval between 74.8 and 76.8, in the region identified as critical to expression of dpp in ps7 of the VM. This abundance of sites in a gene that behaves as a target of Ubx is suggestive of a direct relationship; however, further work is required to determine if these sites are directly responsible for Ubx’s effect on dpp expression.

Nevertheless, in homozygous mutant Ubx embryos, we see significant expression from the RD2 reporter construct in ps7. Residual expression of dpp protein in a UbxΔ28 homozygote was reported by Reuter et al. (1990) who attributed this effect to partial activity by this allele as they suggested by the observation that ectopic expression of Ubx by a heat shock construct is unable to activate dpp in the Abd-A domain (Reuter et al., 1990). These results suggest that a cofactor necessary for initiating dpp’s ps7 gene expression is under negative repression by Abd-A and that the posterior limit of dpp ps7 VM expression depends on several activities of Abd-A.

There are at least two homeobox-containing genes that are expressed in the VM and are candidates for positive coregulators, H2.0 (Barad et al., 1998) and msh-2 (Bodmer et al., 1990). The low level of activity produced by the coregulator(s) alone would not generate sufficient dpp product to mediate biological activity, as all the Ubx loss-of-function alleles we tested appear to be fully penetrant for the loss of the secondary midgut constriction (data not shown).

dpp is required for both the maintenance of its own transcription and that of Ubx in the VM

Using mutations that independently alter specific aspects of dpp’s expression in the VM we have established that both sites of dpp transcript accumulation require dpp protein to achieve correct expression levels. In addition, our data indicates that Ubx autoregulation in ps7 appears to act through dpp. Work by Bienz and coworkers have demonstrated that sequences from –3.1 to +980 are required to provide correct Ubx expression in ps7 of the VM (Müller et al., 1989). We find that Ubx reporter genes containing such sequences express in both ps4 and ps7 of the visceral mesoderm in a pattern remarkably similar to that observed with the dpp RD2 reporter gene, although it should be noted that the initiation of gene expression from 15-1wtUbx appears to occur later than RD2 as judged from the morphology of the anterior midgut. This promiscuous expression in the developing gastric caeca appears to be common among Ubx reporter gene constructs containing approximately this portion of the Ubx gene, both from our results and upon reexamination of the literature (Müller et al., 1989; Irvine et al., 1991). We therefore believe it is not an artifact of a specific construct, or chromosomal location, but reflects aberrant expression due to the absence of other cis-regulatory DNA that causes repression in anterior portions of the embryo. Other workers have described similar ectopic anterior expression in β-galactosidase reporter gene constructs from other regions of the Ubx gene (Simon et al., 1990; Qian et al., 1991), and they also attribute it to the removal of directing a low level of dpp expression in the absence of Ubx.

The second conclusion is based on the observation that ectopic expression of RD2 throughout the posterior VM occurs even in a deficiency for both Ubx and abd-A (UbxΔ108). This expression is spatially identical to that observed in embryos mutant for the abd-A gene alone, although the level of expression is greatly reduced. This result suggests that abd-A acts on dpp by two independent mechanisms: through negative regulation of Ubx itself and through independent negative regulation of a second factor required for dpp’s ps7 VM expression. Thus even in the absence of both Ubx and abd-A, the derepression of the second factor triggers ectopic expression of the reporter gene. The ability of abd-A to act directly on dpp is also suggested by the observation that ectopic expression of Ubx in the VM, even when a heat shock-activated Ubx gene is used (Reuter et al., 1990). Thus, positive transcription factors are required for both the maintenance of its own transcription and that of Ubx in the VM.

Fig. 8. Schematic diagram of a model for interactions of dpp with genes in the VM. Lines with arrowheads indicate positive interactions, lines with bars indicate negative interactions. Our data do not distinguish between abd-A acting through factor X on dpp, or acting on the dpp gene directly, so both possibilities are included (dashed lines).
of cis-regulatory information that represses this expression in vivo. We surmise that short Ubx constructs, lacking the relevant repression domains, would not show the normal repression of Ubx in ps4. In the absence of active repression, the reporter gene might then be capable of responding to some activating network resident in ps4 VM cells. The coincidence of Ubx and dpp expression in the VM suggests that this network includes and absolutely requires the dpp gene. The result obtained supports this idea: VM expression from Ubx reporter genes is eliminated in svh mutant backgrounds lacking dpp gene expression in the VM. Thus the presence of DPP is a requirement for transcription from a Ubx-reporter gene both in ps7, in cells that express endogenous Ubx protein, and in ps4, in cells that do not.

**Autoregulation in the VM**

Autoregulation is invoked in models of pattern formation to account for the stable maintenance of gene expression initiated by transient signals (Meinhardt, 1982). Many Drosophila patterning genes exhibit this characteristic, such as even-skipped (eve; Harding et al. 1989; Goto et al., 1989) fushi tarazu (ftz; Hiromi and Gehring, 1987), Deformed (Dfd; Kuziora and McGinnis, 1988; Bergson and McGinnis, 1990) and Ubx (Bienz and Tremml, 1988; Müller et al., 1989). All these genes are DNA binding proteins that putatively act as transcription factors. Furthermore, all their genes contain sequences that are capable of binding their own gene products in vitro (Jiang et al., 1991; Pick et al., 1990; Regulski et al., 1991; Beachy et al., 1988; von Kessler and Beachy, unpublished observations cited in Beachy, 1990) so it has been tempting to speculate that such forms of autoregulation are direct, and that the products of autoregulatory genes act by binding to their own promoters to positively regulate translation.

This appears to be the case for eve, Dfd, and ftz, where the sites within the autoregulatory elements that bind the respective gene products are required for correct autoregulation (Jiang et al., 1991; Regulski et al., 1991; Schier and Gehring, 1992) although it is conceivable that other homeotic genes with similar site specificity could be the effectors in vivo. In these cases a strong argument can be made for direct positive autoregulation.

The regulation of Ubx in the VM may be different. We have unambiguously demonstrated that dpp is required to maintain Ubx reporter gene transcription in the VM. In ps4 a pathway must exist for dpp to act on Ubx transcription independent of the presence of Ubx protein, which is not expressed in this location. In ps7 there are at least two possibilities: the simpler is that the same pathway obtains in both ps4 and ps7; that is the autoregulation of Ubx in the VM is indirect and acts through dpp and a presently unidentified transcription factor. We have schematized this model in Fig. 8. The other possibility is that dpp acts on Ubx transcription by activating a signal transduction network that post-translationally modifies the Ubx protein itself, allowing it to maintain its own expression. While we cannot distinguish between these two possibilities, we favor the former and raise the possibility that Ubx autoregulation in the VM may be indirect.

In summary, we have demonstrated that the expression of dpp in the VM of the Drosophila embryo is autoregulatory, and that it depends on the interplay of a variety of factors as diagrammed in Fig. 8. While Ubx controls the expression of dpp in ps7, an additional factor (X), whose spatial boundaries are independently controlled, is a necessary cofactor. The expression of Ubx in ps7 requires dpp, but does not absolutely require Ubx itself, invoking yet another regulatory factor (Y). Furthermore, dpp’s autoregulation both in ps4 and in ps7 indicate that dpp’s ability to stimulate its own transcription acts through both a Ubx-independent (ps4) and a Ubx-dependent (ps7) pathway. This may invoke yet another factor resident in ps4 (Z). Experiments with Ubx-heat shock constructs (Reuter et al., 1990), and our own experiments using Ubx-reporter gene constructs indicate that each gene has the capability to induce transcription of the other, and opens the question as to which gene is the primary activator in vivo. We conclude that there is no simple linear gene network regulating midgut morphogenesis, but a complex feedback mechanism maintaining gene expression in this tissue.

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