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TITLE: A Genetic Approach to Identifying Signal Transduction Mechanisms Initiated by Receptors for TGF-B-Related Factors

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decapentaplegic (dpp) is a Transforming Growth Factor beta (TGF-β)-related growth factor that controls multiple developmental processes in Drosophila. To identify components involved in dpp signaling, a modifier screen was carried out for dominant enhancer mutations of a hypomorphic allele of thick veins (tkv), a type I receptor for dpp. New alleles of tkv, punt, Mothers Against dpp (Mad) and Medea (Med), all of which are known to mediate dpp signaling, were recovered. Mutations in the 60A gene, which encodes another TGF-β-related factor in Drosophila, were also recovered. The molecular identities of the new alleles of 60A, Mad and punt were established by DNA sequence analysis. These results also provide the first in vivo evidence for the involvement of 60A in the dpp pathway. We propose that 60A activity is required to maintain optimal signaling by the dpp pathway, possibly by forming biologically active heterodimers with dpp. The enhancer mutations D1 and D2 potentially represent two new loci involved in dpp signaling. The dpp-like phenotypes caused by the enhancer mutations and their genetic interactions with members of the dpp pathway suggest that they are likely to be essential components in the dpp signal transduction pathway.
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INTRODUCTION

The transforming growth factor beta (TGF-β) superfamily consists of a growing number of identified growth factors regulating remarkably diverse aspects of cell life, including cell growth, differentiation, death, adhesion and migration. Since the first discovery of mammalian TGF-β1 16 years ago as a factor capable of inducing anchorage-independent growth of normal rat kidney fibroblasts (Roberts et al., 1981), close to 40 TGF-β-related factors have been identified in organisms as diverse as worms, insects and human. Based on structural and functional relationships, members of this superfamily are subdivided into the prototypic TGF-β family (Massagué, 1990), the Activin/Inhibin family (Ying, 1989), the Bone Morphogenetic Protein (BMP) family (Hogan, 1996b) and other more distantly related factors including the Müllerian Inhibiting Substance (MIS) (Cate et al., 1986), the Growth and Differentiation Factors (GDF) 1, 3, 9 (Lee, 1991; McPherron and Lee, 1993) and the Glial-Derived Neurotrophic Factor (GDNF) (Lin et al., 1993). The BMPs constitute the largest subgroup, including mammalian BMPs 2-13, Nodal and GDF-5; chicken dorsalin 1; Xenopus Vgr-1 and Nodal related XNr1-3; and Drosophila decapentaplegic (dpp), 60A and screw (scw) (Hogan, 1996b).

Even though TGF-βs were initially identified by their ability to transform certain cell lines, they are best known for their potent antiproliferative effects on cells from various lineages (Moses et al., 1990; Massagué, 1990). Recent studies revealed that TGF-β partly acts through transcriptional activation of genes whose products directly interfere with cell cycle progression. TGF-β treatment increases the protein levels of p15, p21 and p27, members of the families of cyclin-dependent kinase (cdk) inhibitors (Sherr and Roberts, 1995). These inhibitors inactivate G1 cdks, resulting in hypophosphorylation of the retinoblastoma gene product, Rb and subsequent cell cycle arrest at the G1 to S transition (Reynisdottir et al., 1995; Hannon and Beach, 1994; Datto et al., 1995). The antiproliferative effect of TGF-βs is also mediated by rapid inhibition of genes that participate in G1 progression, such as members of the Myc family (Alexandrow and Moses, 1995). The observations that various tumor-derived cell lines are unresponsive to TGF-β (Fynan and Reiss, 1993) and that in certain epidermal tumors, the malignancy is associated with defects in autocrine TGF-β production (Glick et al., 1994) suggest that TGF-β signaling has tumor-suppressing functions.

In addition to regulating cell growth, TGF-β-related factors, BMPs in particular, also play pivotal roles in development. The founding members of the BMP family were first identified based on their ability to stimulate ectopic bone formation when implanted in experimental animals (Wozney et al., 1988). In fact, BMPs regulate various developmental processes by acting as morphogens to pattern cellular fields in a concentration dependent manner, as inducers of cell fates across germ layers and as messengers for the so-called community effect that coordinates
the differentiation of groups of cells (Moses and Serra, 1996; Hogan et al., 1994; Hogan, 1996a; Hogan, 1996b). For example, in Xenopus embryonic tissues, cells express different genes depending on their distance from a source of activin, suggesting the operation of a morphogen gradient in vertebrates (Gurdon et al., 1994). Similar concentration-dependent patterning of the Xenopus gastrula ectoderm by BMP-4 has also been demonstrated (Wilson et al., 1997). In the chick limb, ectopic expression of BMP-2 in the anterior mesenchyme induces FGF-4 expression in the overlying apical ectodermal ridge (AER) (Duprez et al., 1996). In mice, BMP-2, -4, and -7 are all expressed in the mesenchyme as well as in the AER of the developing limb bud, where they may signal to pattern the underlying mesenchymal progress zone (Hogan, 1996b). Similar cross tissue interactions mediated by BMPs are also important for mammalian tooth morphogenesis (Vaahtokari et al., 1996). Recently, several spontaneous mutations were shown to affect murine BMP genes, including BMP-5 (short ear), GDF-5 (brachypodism) and nodal. In addition, five more BMP genes have been inactivated by targeted mutagenesis, including BMP-2, -4, -7 and -8B. Analysis of homozygous phenotypes has provided genetic evidence for the importance of BMPs in the establishment of the basic embryonic body plan and the development of nearly all organs and tissues (see references in Hogan, 1996b).

In a signaling system involving multiple interacting signaling components, a great diversity of cellular responses can be achieved through combinatorial actions such as the formation of heterodimers between distinct ligand monomers at sites of overlapping expression. One example of how the composition of a TGF-β-like molecule can drastically alter its activity is provided by the inhibins and activins, which share common subunits. Activins that are homodimers of β subunits have opposite biological activities to the inhibins, which are heterodimers of α and β subunits (Ling et al., 1986; Petraglia et al., 1989). In addition to having antagonistic functions, heterodimers can also function synergistically with the homodimers. For example, heterodimers of Xenopus BMP-4 and -7 were shown to be more potent in bone and mesoderm formation assays than either of the homodimers (Aono et al., 1995; Suzuki et al., 1997). In mice, the overlapping spatial expression of several BMPs suggests that these growth factors may combinatorially regulate cell fate specification (Lyons et al., 1995).

The biosynthesis and maturation processes of TGF-β-related factors are tightly regulated and, to some extent, contribute to their versatile functions. These factors are synthesized as large precursor molecules that undergo proteolytic cleavages at a cluster of dibasic residues to yield mature bioactive carboxy termini (Roberts and Sporn, 1990; Panganiban et al., 1990a; Doctor et al., 1992). Most members of this family have seven conserved cysteines in the carboxy ligand domain. Structural analysis of TGF-β2 (Daopin et al., 1992) and BMP-7 (Griffith et al., 1996) revealed that six of the cysteines form a cysteine knot through intramolecular disulfide bonds and that the seventh cysteine participates in an intermolecular disulfide bond to form a dimer. Dimerizing with different partners can drastically alter the ligands'
biological activity, as best exemplified by the activins and inhibins. Activins, which are homodimers of β subunits have the opposite effect on the regulation of follicle stimulating hormone release than inhibins, which are heterodimers of α and β subunits (Ying, 1989). Upon secretion, the access of these growth factors to cell surface receptors is regulated by their complex interactions with various extracellular proteins (Miyazono et al., 1993). For example, the binding of activin by follistatin, a soluble protein in ovary, prevents access of activin to its receptors (Hemmati-Brivanlou et al., 1994). The binding of TGF-β2 to betaglycan, a membrane proteoglycan, profoundly increases its affinity for the signaling receptors (Lopez et al., 1993). However, when expressed in a soluble form or released from the cell surface by the action of an endogenous protease, betaglycan sequesters TGF-β and acts as a potent antagonist of TGF-β binding to cell surface receptors (Lopez et al., 1994).

Cross-linking experiments using radiolabeled ligands and expression cloning approaches have identified a novel class of transmembrane serine/threonine kinases as the major cell surface receptors for TGF-β-related growth factors (reviewed by Attisano et al., 1994; Massagué and Weis-Garcia, 1996). These receptors are divided into the type I and type II families based on their distinct primary sequences and functions. Studies of the receptor binding profile of a panel of mutant epithelial cell lines resistant to TGF-β-induced growth arrest suggest that both types of receptors are required for signaling (Boyd and Massagué, 1989; Laiho et al., 1990; Laiho et al., 1991). Further biochemical and functional analyses of these cell lines have established the mechanism of receptor activation for TGF-β (Wrana et al., 1994). The type II receptor is the principle ligand binding component and has a constitutively active kinase activity. Upon ligand binding by the type II receptor, the type I receptor is recruited to form a heteromeric complex with the type II receptor, which activates the type I receptor by phosphorylating it in a conserved cytoplasmic domain rich in glycine and serine (the GS domain). This mechanism places the type I receptor downstream of the type II receptor during signal transduction. Numerous studies have confirmed the generality of this mechanism. Some variation resides in the ligand binding properties of BMP receptors, in which case high affinity ligand binding requires cooperation between both types of receptors (see references in Attisano and Wrana, 1996).

The presence of multiple ligands, receptors and heteromeric receptor complexes highlights the combinatorial nature of TGF-β-related signaling. There is evidence that different downstream responses can be specified by distinct signal transducers. For example, ActRII and ActRIIB, two mammalian activin type II receptors, are also capable of binding BMP-7 under physiological conditions and of transducing activin-like effects (Yamashita et al., 1995). Meanwhile, the recruitment of distinct activin type I receptors, ActRI or ActRIB into a complex with ActRIIB elicits different cellular responses in the presence of activin, suggesting that type I receptors play a central role in specifying downstream effects (Yamashita et al., 1995).
This function of the type I receptor is likely linked to its kinase activity since type I receptors with highly related kinase domains transduce similar cellular responses even though the ligand and the type II receptor partners are distinct (Carcamo et al., 1994).

Since the identification of the receptors for TGF-β-related growth factors, much effort has been focused on identifying components essential for relaying the signal from cell surface to nucleus. Several groups have used the yeast two hybrid system (Fields and Song, 1989) to look for proteins that directly interact with the cytoplasmic domains of TGF-β receptors. The TGF-β type I receptor (TβRI) interacting proteins identified include FK506/rapamycin binding protein (FKBP12) (Wang et al., 1994) and farnesyl transferase-α (FT-α) (Kawabata et al., 1995; Wang et al., 1996a; Ventura et al., 1996). TGFβ-receptor interacting protein-1 (TRIP-1) (Chen et al., 1995) and apolipoprotein/clusterin (Reddy et al., 1996) were identified based on their interactions with the TGF-β type II receptor (TβRII). FKBP12 has been postulated to function as a negative regulator of the TGF-β pathway based on the observation that blocking its interaction with TβRI using non-functional derivatives of FK506 leads to an enhancement of TGF-β signaling (Wang et al., 1996b). Since mutations in TβRI that disrupt FKBP12 binding do not affect the signaling capacity of the receptor (Charng et al., 1996), FKBP12 is more likely a modulator rather than a direct signal transducer of the pathway. The physiological relevance of other interacting proteins mentioned above in TGF-β signaling remains uncertain. TAK1 (TGF-β-activated kinase) was identified in a screen for members of the mammalian mitogen-activated protein kinase kinase family (MAPKKK) capable of activating a yeast pheromone-induced MAPK pathway (Yamaguchi et al., 1995). The activation of TAK1 leads to a typical TGF-β mediated transcriptional response and requires the kinase activity of TAK1 (Yamaguchi et al., 1995). Subsequently, a yeast two hybrid screen identified TAB1, a novel protein which interacts with TAK1 and enhances its kinase activity (Shibuya et al., 1996). These data suggest that TAK1 and TAB1 may mediate TGF-β signaling, but since the kinase activity of TAK1 is stimulated by TGF-β as well as BMP-4 (Yamaguchi et al., 1995), their precise role in TGF-β signaling requires further investigation.

In Drosophila, a screen for maternal enhancer mutations of a hypomorphic dpp allele allowed identification of Mothers against dpp (Mad) and Medea (Med), which are required in many dpp-dependent developmental processes (Raftery et al., 1995; Sekelsky et al., 1995). Mad is required in dpp responding cells (Newfeld et al., 1996), suggesting that Mad plays a role in mediating dpp signaling. Mutations in Mad suppress dominant phenotypes generated by a constitutively active type I dpp receptor, placing Mad downstream of the receptors (Hoodless et al., 1996; Wiersdorff et al., 1996; Newfeld et al., 1997). In C. elegans, attempts to recover mutations affecting dauer larvae formation, a process controlled by BMP-like factors, identified sma-2, -3, and -4, which encode proteins homologous to Mad (Savage et al., 1996).
Mad homologues were soon identified in frog, mouse and human. These Mad-related proteins constitute the Smad family. Extensive biochemical and genetic analysis revealed that Smads are key cytoplasmic signal transducers in TGF-β-related pathways, linking receptor activation at the cell surface and target gene expression changes in the nucleus (reviewed by Derynck and Zhang, 1996; Massagué, 1996; Wrana and Attisano, 1996; Wrana and Pawson, 1997). Vertebrate Smad1 through 7 fall into three functionally distinct categories. Smad1, 2, 3 and probably Smad5 are receptor-regulated-Smads which associate directly with, and are phosphorylated within a conserved C terminal SSXS domain by, activated type I receptors (Hoodless et al., 1996; Kretzschmar et al., 1997; Macias-Silva et al., 1996; Zhang et al., 1996). The phosphorylated Smads then form a complex with Smad4 and accumulate in the nucleus (Lagna et al., 1996; Macias-Silva et al., 1996; Hoodless et al., 1996; Zhang et al., 1997; Wu et al., 1997) where they can function as transcriptional activators. In Drosophila, Mad can directly bind the enhancer of vestigial and mediate its activation by dpp (Kim et al., 1997). In Xenopus, Smad2 interacts with the fork-head-DNA-binding protein, FAST1, to activate the activin responsive element in the mix.2 gene (Chen et al., 1996). The receptor-regulated-Smads seem to specify the biological responses in that Smad1 and 5 function in BMP signaling (Hoodless et al., 1996; Kretzschmar et al., 1997; Liu et al., 1996; Thomsen, 1996) while Smad2 and 3 function in TGF-β and activin signaling (Baker and Harland, 1996; Eppert et al., 1996; Graff et al., 1996; Lechleider et al., 1996; Macias-Silva et al., 1996; Yingling et al., 1996). Structural and mutational analysis of the conserved C terminal domain of Smad4 suggests that Smads form homotrimers and that the phosphorylated receptor-activated-Smads may undergo a conformational change which enables them to form a heterohexamer with the homotrimeric Smad4 protein (Hata et al., 1997; Shi et al., 1997). More recently, a new class of Smads including mammalian Smad6 and 7, the Drosophila Daughters against dpp (Dad) and the C. elegans Daf-3 have been found to function as antagonists in TGF-β-related signaling (Hayashi et al., 1997; Nakao et al., 1997; Imamura et al., 1997; Tsuneizumi et al., 1997; Patterson et al., 1997). Thus, Smad proteins can both positively and negatively regulate TGF-β-related signaling. Consistent with the notion that the TGF-β pathway has a tumor-suppressing function, mutations in Smads are associated with various types of tumors (Hahn et al., 1996; Schutte et al., 1996; Eppert et al., 1996; Thiagalingam et al., 1996).

Given the remarkably diverse physiological functions of the TGF-β-related factors, understanding their mode of action should provide new strategies to treat cancer, to repair damaged tissues and to modulate development. However, the vast number of TGF-β-related factors in vertebrates and the potential interactions and functional redundancy among related factors make assigning precise roles to each factor a daunting task. The identification of TGF-β-related pathways in Drosophila enabled us to take advantage of the power of genetics in this relatively simple organism to begin addressing some of the fundamental questions regarding TGF-β-related signal transduction.
In *Drosophila*, the TGF-β-related factors are represented by the *decapentaplegic (dpp)*, *60A* and *screw (scw)* gene products (Padgett *et al.*, 1987; Doctor *et al.*, 1992; Wharton *et al.*, 1991; Arora *et al.*, 1994). All three are members of the BMP family. At the amino acid level, *dpp* is 75% identical to mammalian BMP-2 and -4 and *60A* is 70% identical to BMP-5 through -8 (Kingsley, 1994a). When implanted subcutaneously in rats, Dpp and 60A proteins are able to induce ectopic bone formation (Sampath *et al.*, 1993). Furthermore, human BMP-4 is able to rescue the dorsal/ventral patterning defects of *dpp* mutant embryos (Padgett *et al.*, 1993), demonstrating the functional conservation of the dpp/BMP-4 pathway throughout evolution. Scw proteins have been proposed to enhance *dpp* activity during early embryogenesis by forming heterodimers with Dpp (Arora *et al.*, 1994). The role of 60A in development has remained unclear due to the lack of mutations in the 60A gene.

*dpp* has a dynamic expression pattern correlating with its multiple functions throughout *Drosophila* development. At the blastoderm stage, *dpp* transcripts are restricted dorsally (St. Johnston and Gelbart, 1987) where *dpp* functions as a morphogen to specify distinct dorsal structures (Ferguson and Anderson, 1992b; 1992a; Wharton *et al.*, 1993). Later during embryogenesis, *dpp* is expressed in the ectoderm (Jackson and Hoffmann, 1994), where it induces differentiation of the dorsal mesoderm (Staehling-Hampton *et al.*, 1994). In the visceral mesoderm, *dpp* is expressed in discrete domains to regulate the expression of several homeotic genes and to induce the expression of the homeotic gene *labial (lab)* in the underlying endoderm (Immerglück *et al.*, 1990; Panganiban *et al.*, 1990b). *dpp* is also expressed in specific positions in the larval imaginal discs (Masucci *et al.*, 1990) to control the proliferation and patterning of adult appendages (reviewed by Brook *et al.*, 1996).

In *Drosophila*, two type I receptors encoded by *saxophone (sax)* and *thick veins (tkv)* and one type II receptor encoded by the *punt* gene have been shown to be functional *dpp* receptors (Penton *et al.*, 1994; Brummel *et al.*, 1994; Nellen *et al.*, 1994; Ruberte *et al.*, 1995; Letsou *et al.*, 1995; Xie *et al.*, 1994). *Mothers against dpp (Mad)* and *schnurri (shn)* are two factors identified through genetic interactions with *dpp* (Raftery *et al.*, 1995; Sekelsky *et al.*, 1995; Staehling-Hampton *et al.*, 1995; Grieder *et al.*, 1995; Arora *et al.*, 1995). *shn* encodes a protein related to human zinc finger transcription factor PRDII/MBPI/HIV-EPI (Staehling-Hampton *et al.*, 1995; Arora *et al.*, 1995; Grieder *et al.*, 1995). Mad-related proteins (Smads) have been isolated from a wide range of distantly related organisms. Genetic and biochemical evidence indicates that they are key signal transducers, linking events between receptor activation and changes in target gene expression (Derynck and Zhang, 1996; Massagué, 1996; Wrana and Pawson, 1997).

The rapid advance made in understanding the mechanisms by which TGF-β-related signals are relayed from cell surface to nucleus clearly demonstrates the power of genetic approaches to dissecting a biological pathway. Modifier genetics
utilizes the synergistic interactions between components in the same biological pathway. In such a system, if the signaling output is just below the optimal level and produces a visible phenotype, then the pathway is likely to be sensitive to a reduction in the levels of other essential components in the same pathway, resulting in an enhanced or suppressed phenotype, depending on whether the component is a positive or negative regulator of the pathway. In *Drosophila*, modifier genetics has been successfully used to isolate essential components in the *sevenless* receptor tyrosine kinase pathway (reviewed by Hafen *et al.*, 1993) and in the cytoplasmic *Abelson* tyrosine kinase pathway (reviewed by Hoffmann, 1991). In both cases, the characterization of second-site modifiers has been instrumental in understanding how similar pathways may function in higher organisms.

The dosage sensitive nature of the *dpp* signaling pathway prompted us to use modifier genetics to isolate new components in the pathway. Using the phenotypes generated by a hypomorphic *dpp* receptor as a genetic background, we screened for enhancer mutations that exacerbate the phenotype. New alleles of many genes known to be involved in the *dpp* pathway were recovered, as well as mutations in 60A, a BMP-7 homologue, whose function was not previously known.
EXPERIMENTAL METHODS

Source of antibodies

Anti-Scr, anti-Ubx, anti-Lab antibodies were gifts of Dr. Matthew Scott, Stanford University. Anti-Wg antibody was a gift of Dr. Roel Nusse, Stanford University. Anti-Dpp antibody was described in Panganiban et al. (1990a). The mouse monoclonal anti-En antibody mAb 4F11 were described in Patel et al (1989). The rabbit anti-β-galactosidase antibodies were purchased from Rockland. The mouse anti-β-galactosidase antibodies were purchased from Promega. The biotin conjugated secondary antibodies, the horse radish peroxidase (HRP) conjugated secondary antibodies and the HRP conjugated streptavidin were purchased either from Vector Laboratories Inc., Boehringer Mannheim, GIBCO BRL or Jackson ImmunoResearch.

Anti-Dpp staining

Anti-Dpp staining was done according to the procedures described in Panganiban et al. (1990b) with minor modifications. Briefly, staged embryos were dechorionated in 50% bleach and were then fixed in 1 part heptane and 1 part 4% paraformaldehyde in phosphate buffered saline (PBS) for 20 minutes. The fixed embryos were devitellinized in 1 part heptane and 1 part methanol, followed by rinses in methanol. The embryos were used immediately for staining. After rehydration in PBS, the embryos were blocked in PBSTB (PBS with 0.1% Triton X-100 and 0.2% bovine serum albumin (BSA)) and 5% normal goat serum (NGS) for at least 3 hours. The embryos were then incubated with the pre-absorbed rabbit anti-Dpp antibodies (1 μg/ml) in PBSTB with 5% NGS at 4°C overnight. The stained embryos were washed for at least 1 hour at room temperature in PBSTB with at least four changes of wash solutions and blocked for half an hour in PBSTB with 5% NGS. Embryos were then incubated with biotinylated anti-rabbit-IgG secondary antibodies (1:300) in PBSTB with 5% NGS at 4°C overnight. After washing with PBSTB as described for the primary antibody incubation, the embryos were incubated with HRP-conjugated streptavidin (1:300) in PBSTB plus 5% NGS for at least one hour at room temperature. The embryos were washed as described above. The peroxidase reactions were done in 1X diaminobenzidine (DAB) solution (0.25 mg/ml in 100 mM Tris, PH 7.5) with 0.003% H2O2 and 0.08% of CoCl2 ad NiCl2. The reaction was monitored under a dissecting scope and stopped by washing with PBSTB when the background appeared lightly stained. After thorough washing, the stained embryos were dehydrated in an ethanol series and mounted in 80% glycerol. The embryos were visualized with Nomarski optics and pictures were taken with Kodak Royal Gold 25 film.

Anti-Lab, anti-Wg, anti-Scr, anti-Ubx and anti-Antp stainings

Anti-Lab (1:200), anti-Wg (1:300), anti-Scr (1:10), anti-Ubx (1:5) and anti-Antp (1:5) stainings were essentially the same as anti-Dpp staining except that the embryos were fixed in 1 part of heptane and 1 part 4% formaldehyde in PMG buffer (0.1 M PIPES, 2 mM EGTA and 1 mM MgSO4). For anti-Wg staining, lowering the NGS
concentration from 5% to 1% gave better staining results. For anti-Lab and anti-Wg stainings, a biotin conjugated secondary antibody and an HRP conjugated streptavidin were used. For anti-Scr, anti-Ubx and anti-Antp stainings, an HRP conjugated anti-mouse IgG antibody was used.

**Anti-β-galactosidase double-staining**

Sequential staining reactions were done for embryos needed to be double-stained with the anti-β-galactosidase antibody. Briefly, the embryos stained with the first antibodies were developed in DAB solution containing nickel and cobalt ions as described above. The stained embryos were washed extensively and blocked as described and then incubated with either rabbit anti-β-galactosidase antibodies (1:800) or mouse anti-β-galactosidase antibodies (1:300). After incubation with appropriate HRP conjugated secondary antibodies, the anti-β-galactosidase antibodies were detected by the oxidation of DAB in the absence of nickel and cobalt ions.

**Anti-Engrailed and anti-β-galactosidase stainings**

Imaginal discs were dissected in PBS and fixed in 2% formaldehyde in PIPES buffer (0.1 M PIPES (PH 6.9), 2 mM MgSO4, 1 mM EGTA, 1% NP-40) for 20 minutes, washed in the wash buffer (PBS with 0.3% Triton X-100 (PT) and 1 mg/ml BSA) and blocked in PTB buffer (PT with 5 mg/ml BSA) for 1 hour. Mouse monoclonal antibody, mAb 4F11 for Engrailed protein (Patel et al., 1989) (1:25 dilution) and rabbit anti-βgalactosidase (1:800 dilution, Rockland) for wg-lacZ were used in PTB buffer at 4°C overnight. The discs were washed in the wash buffer for at least one hour with frequent changes. Secondary antibodies used were FITC conjugated anti-mouse IgG and rhodamine conjugated anti-rabbit IgG (both at 1:200 dilution, Jackson ImmunoResearch). After washes, the stained discs were mounted in the Glycerol Mount (30% glycerol, 5 mM Tris (PH 8.8), 150 mM NaCl, 0.02% NaN3). To prevent photo bleaching, PDA (p-Phenylenedianime P1519, Sigma) was added to the Glycerol Mount at a final concentration of 0.5 mg/ml). The images were collected using the confocal microscope at the W. M. Keck Foundation neural imaging laboratory.

**Drosophila stocks**

*Drosophila* stocks were cultured on standard cornmeal yeast extract and sucrose medium at 25°C, unless otherwise specified. *Canton S.* was used as the wild type stock. *sax⁵* was described in Twombly et al (1996). *Mad⁴* is described in Sekelsky et al (1995). *Med⁴* was described in Raftery et al (1995). *p[w⁺UAS-tkvQD]⁴* was described in Nellen et al (1996). The vestigial-Gal4181 line has a vestigial intron 2 enhancer (Williams et al., 1994) that drives the expression of Gal4 proteins in the anterior and posterior hinge regions of the wing disc (Morimura and Hoffmann, unpublished). All other mutants and chromosomes are referenced or described in Linsley and Zimm (1992). Mutant stocks were obtained from
Bloomington, Umea stock centers, Berkeley Drosophila genome project and from A. Letsou, V. Twombly, M. Singer, W. Gelbart and K. Wharton.

N-ethyl-N-nitrosourea (ENU) mutagenesis

One gram of ENU (Sigma, ISOPAC) was dissolved in 10 ml of 95% ethanol and diluted with 90 ml of the phosphate/citrate buffer (1 M Na₂HPO₄, 0.05 M Na₃C₆H₅O₇, PH 5.0, adjusted with phosphoric acid). An appropriate amount of the stock ENU solution (10 mg/ml) was further diluted with 1% sucrose solution to give a final concentration of 0.4 mg/ml (3.4 mM). All manipulations were performed in a fume hood and the ENU solution was made fresh. 1M NaOH solution was used to soak the containers and the waste overnight to neutralize the ENU.

Initial attempts to recover modifiers of tkv⁶ in an F1 screen were not successful because of greatly reduced viability and fertility of the flies with enhanced phenotypes. Thus an F2 screen was carried out. pr cn was recombined onto the tkv⁶ chromosome and the stock was made to be isogenic for the second and third chromosomes. Young tkv⁶ pr cn/CyO males were collected and aged at 25°C for 5 days or at room temperature for 7 days. The males were starved for at least 12 hours prior to feeding 3.4 mM ENU in 1% sucrose solution for 18 to 24 hours. The mutagenized males were mated to females that have a reciprocal translocation between CyO and TM6,B (T(2;3)) to force the cosegregation of the second and third chromosome. The males were removed after three days of mating to prevent clustering effect. Individual male progeny were mated to tkv⁶/CyO; TM2/TM6,B females. Enhancement of the imaginal disc derived structures were screened in the progeny homozygous for tkv⁶. The chromosomes with the enhancer mutations were recovered from the siblings of the enhanced progeny and crossed to tkv⁶/CyO; TM2/TM6,B females for retesting and to establish balanced stocks.

The number of complementation groups were determined from inter se crosses among enhancers. For enhancers on the second chromosome, a tkv transgene on the third chromosome, P[w+tkv]1C-10 was used to compensate for the tkv⁶ mutation. Allelism with known mutations was established by genetic non-complementation and by meiotic and deletion mapping. The Sp Bl L⁷m Bc Pu² Pin⁷ chromosome was used for meiotic mapping of the enhancers on the second chromosome. The ru h th st cu sr e ca chromosome was used to map the enhancers on the third chromosome. The "deletion kit" from Bloomington Stock center (Indiana) was used for deletion mapping.

Gamma ray mutagenesis

Non-isogenized Canton S. males were used. The males were aged as described for the ENU mutagenesis and exposed to 4,500 rads of γ-rays from a 137Cs source (Mark I Irradiator). The mutagenized males were allowed to mate to
In(2LR)Gla Bc/CyO females for 3 days. Individual Cy male progeny from this cross were mated to either tkv\(^6\) pr cn D1/CyO, tkv\(^6\) pr cn D2/CyO or tkv\(^6\) pr cn D4/CyO females. The crosses were scored for the absence of Cy progeny. The lethal mutations were recovered from siblings and crossed to the appropriate tester stocks for retesting and to establish balanced stocks.

**P element mutagenesis**

w; P[w\(^+\)]l(2)k13214/CyO (Berkeley Drosophila genome Project) females were mated to w, Sp/CyO; Sb \(\Delta 2-3/+\) males. w; P[w\(^+\)]l(2)k13214/CyO; Sb \(\Delta 2-3/+\) male progeny were then crossed to w; In(2LR)Gla Bc/CyO females. Individual w\(^+\), Sb\(^+\) and Cy male progeny were mated to w; tkv\(^6\) pr cn D4/CyO; TM2/TM6,B females. The crosses were screened for the absence of Cy progeny. The lethal mutations were recovered from the siblings and were mated to w; tkv\(^6\) pr cn D4/CyO; TM2/TM6,B females for re-testing and to establish balanced stocks.

**Genetic Crosses**

wg-lacZ was recombined onto the tkv\(^7\) chromosome. w; tkv\(^7\) wg-lacZ/Df(2L) tkv2; P[w\(^+\); tkv] 1A-8/TM2 adult flies were generated by mating w; tkv\(^7\) wg-lacZ/In(2LR) Gla Bc; P[w\(^+\); tkv] 1A-8/TM2 females to Df(2L) tkv2/In(2LR) Gla, Bc; P[w\(^+\); tkv] 1A-8/TM2 males. The w; tkv\(^7\) wg-lacZ/Df(2L) tkv2; P[w\(^+\); tkv] 1A-8/TM2 larvae were identified by the absence of the Bc marker.

For enhancement mapping of the enhancers on the second chromosome, tkv\(^6\) pr cn */CyO (asterisk indicates the enhancer mutation) males were crossed to Sp Bl L\(\text{rm}\) Bc Pt\(^2\) Pin\(B\) /CyO females. The resulting Cy\(^+\) female progeny were crossed to In(2LR)Gla Bc/CyO males. Males that were Sp\(^+\) and balanced over CyO were collected to maximize the chances of retaining the tkv\(^6\) mutation and individually crossed to tkv\(^6\)/CyO; TM2/TM6,B females. The Cy\(^+\) progeny were scored for dominant markers and the presence or absence of enhancement effects.

For mapping the lethality of D2 over the \(\gamma\)-ray allele D2-1, tkv\(^6\) pr cn D2/CyO males were crossed to Canton S. females. The resulting Cy\(^+\) females were crossed to In(2LR)Gla Bc/CyO males. Preliminary meiotic mapping based on enhancement of tkv\(^6\) indicated that D2 is between Bl and L\(\text{rm}\), so two nearby markers, pr and cn were used for the lethality mapping. Using the pr cn markers present on the CyO chromosome, the Cy male progeny were grouped by genotype with regard to the pr and cn loci (i.e. grouped by pr\(^+\) cn\(^+\); pr cn\(^+\); pr\(^+\) cn or pr cn). These males were then individually crossed to D2-1/CyO females and the crosses were scored for the presence or absence of Cy progeny. The distance between the pr, cn and the lethal locus was calculated by the recombination frequency observed.
Adult flies with the enhanced imaginal disc phenotypes were generated by mating tkv\textsuperscript{6} pr cn\textsuperscript{*}/CyO (asterisk indicates the enhancer mutations) males to tkv\textsuperscript{6} pr cn/CyO females. The tkv\textsuperscript{6} Mad\textsuperscript{D24}/+ embryos were generated for cuticle preparations by mating tkv\textsuperscript{6} Mad\textsuperscript{D24}/CyO females to Canton S. males. The w; tkv\textsuperscript{6} pr cn Mad\textsuperscript{D3} or tkv\textsuperscript{6} pr cn D1/Gal4-vg\textsuperscript{181}; P[w\textsuperscript{+} UAS-tkv\textsuperscript{QD}]/+ adult flies were generated by mating w; Gal4-vg\textsuperscript{181}; + females to w; tkv\textsuperscript{6} pr cn Mad\textsuperscript{D3} or tkv\textsuperscript{6} pr cn D1/In(2LR)Gla Bc; P[w\textsuperscript{+} UAS-tkv\textsuperscript{QD}]/TM2 males.

The 60\textsuperscript{A}D\textsuperscript{4}/60\textsuperscript{A}D\textsuperscript{8} larvae were generated by mating 60\textsuperscript{A}D\textsuperscript{4}/CyO-HShid males to 60\textsuperscript{A}D\textsuperscript{8}/CyO-HShid females. The cross was heat shocked on day 3 and day 4 for 2 hours at 37°C to kill progeny classes inheriting the CyO-HShid chromosome. Escapers were observed when the heat shock was done after the early second instar stage. The In(2LR)Gla Bc chromosome was not used for identification of homozygous mutants because it does not suppress recombination on distal 2R where 60A is located.

The embryos used for antibody staining were generated by mating the appropriate mutants balanced over a CyO chromosome marked with an elav-lacZ enhancer trap. After double staining with the antibodies of interest and anti-β-galactosidase antibody, the homozygous mutants were identified by the absence of anti-β-galactosidase staining.

**Preparation of larval gut, cuticle and adult appendages**
Larval guts were dissected and mounted according to Masucci and Hoffmann (1993). Briefly, third instar larvae were dissected and fixed in 50% ethanol for 5 to 10 minutes. Larval guts were then mounted in 90% glycerol and photographed using bright field optics. Cuticles were prepared as described previously (Struhl, 1989). Briefly, dechorionated, devitellinized embryos or first instar larvae were fixed in 1:4 glycerol: acetic acid for one hour at 60°C. The embryos were mounted in Hoyer’s mounting media (Ashburner, 1989) and allowed to clear overnight at 60°C. Cuticles were photographed under phase contrast optics using Kodak Techpan high contrast film or Kodak Royal Gold 25 film. Wings and legs were mounted in Gary’s magic mounting media (Ashburner, 1989) and photographed under bright field optics using Kodak Royal Gold 25 film.

**Molecular cloning procedures**
Unless otherwise described, the molecular cloning procedures were performed following standard protocols as described in Sambrook et al (1989).

**Cosmid library screening**
The probes used for the library screening were labeled with α-\textsuperscript{32}P-dCTP using the random priming method. Briefly, 20 to 100 ng of DNA was denatured with 2 μg of random nanomer primers in a boiling water bath for 5 minutes, followed by
incubation on ice for 2 minutes to allow annealing to occur. The annealed template DNA and primers were incubated with 50 mM dATP, dTTP, dGTP mix, 50 μCi of α-32p-dCTP, 1X Klenow reaction Buffer and 2-3 units of Klenow enzyme in a total volume of 25 μl for 25 minutes at 37°C. The unincorporated dNTPs were removed using the NucTrap Probe Purification Columns (Stratagene). The probes were denatured in 0.2 N NaOH at room temperature for at least 10 minutes before use. Labeled probes were used immediately or stored at -20°C.

The iso-1 cosmid genomic library (gift of Dr. Tamkun, University of California, Santa Cruz) was plated onto LB/Amp (40 μg/ml) plates at a density of 3,000 to 5,000 colonies/150 mm plate. After overnight growth, the plates were chilled at 4°C for at least an hour to prevent colonies from streaking during lifting procedures. The colonies were lifted onto Amersham N+ Hybond nylon membranes. The filters were then denatured for 5 minutes in denaturing solution (0.5 M NaOH, 1.5 M NaCl) and neutralized for 5 minutes in the neutralizing solution (0.5 M Tris PH 7.5, 1.5 M NaCl). After neutralization, the filters were cross-linked using a Stratagene UV-Crosslinker on automatic setting. The cross-linked filters were washed in low stringency wash buffer (2XSSC, 1% SDS) for at least 1 hour at 65°C and pre-hybridized in the hybridization buffer (120 mM Na2HPO4, PH 7.2, 250 mM NaCl, 7% SDS, 10% PEG 8000, 30% formamide) for at 5 minutes at 37°C. Following pre-hybridization, the filters were incubated with fresh hybridization buffer and the labeled probes (1X10^6 CPM/ml) overnight at 37°C. After hybridization, the filters were first washed for half an hour in the low stringency wash buffer at 37°C with 2 changes, followed by washing for 2 hours in the high stringency buffer (0.1XSSC, 1% SDS) at 65°C with 4 changes. Autoradiography was done at -80°C with an intensifying screen.

A 5 kb RI rescued fragment from 60ΔD4-P2 genomic DNA was used as a probe to screen about 30,000 independent clones and four positive clones were isolated. Restriction enzyme digest and Southern analysis using internal fragments of the cosmid allowed the construction of the maps of the tkv and 60A genomic region.

Genomic Southern analysis

Genomic DNA was isolated according to the method of Ashburner (1989). The DNA was digested to completion with appropriate restriction enzymes and separated on agarose gels. Gels were treated with 0.2 M HCl for 15 minutes followed by denaturation in 0.4 N NaOH for 45 minutes. The DNA was then transferred to the Zeta Probe nylon membranes (BioRad) in 0.4 N NaOH using a vacuum blotter (Pharmacia). The hybridization and washing conditions were essentially the same as described for library screenings.

Plasmid rescue

Twenty-five to 30 flies were homogenized in 700 μl of homogenization buffer (HB) (0.2 M sucrose, 0.1 M Tris, PH 9.2, 50 mM EDTA, 0.5% SDS) and incubated at 68°C for 10 minutes. One hundred and five μl of 8 M KOAc was added and the
homogenate was incubated on ice for 15 minutes. After spinning down the debris, the supernatant was collected and the DNA was precipitated for at least 1 hour at room temperature after adding one volume of isopropanol. The DNA was resuspended in TE and NaCl was added to a final concentration of 0.1 M. The DNA solution was extracted with phenol/chloroform and precipitated again by adding 2 volumes of ethanol. The genomic DNA was resuspended to a concentration of 1 fly equivalent/μl (FE). Five FE of genomic DNA was digested with the appropriate restriction enzymes in the presence of RNase (EcoRI was used to recover the genomic DNA on one end of the P insertion; BgIII was used to recover genomic DNA on the other end of the insertion. For a map of the P-lacZW vector, see (Bier et al., 1989)). The restriction enzyme reaction was phenol/chloroform extracted and the DNA was precipitated and briefly dried. The intramolecular ligation was set up in a 1 ml volume with 1 unit of the ligase (Boehringer Mannheim). Following incubation overnight at 16°C, the ligation reaction mixture was phenol/chloroform extracted and precipitated by adding 1 volume of PEG solution (1.6 M NaCl, 13% PEG 8000) and resuspended in 10 μl of ddH2O. Two μl of the resuspended DNA was used for electroporating into DH5α E. coli using the Zapper Electroporation Unit (UW Medical Electronics Lab) and transformed bacteria were plated onto LB/AMP (50 μg/ml) plates.

Sequencing of mutant alleles
Total RNA was isolated from heterozygous Mad and punt females and heterozygous 60A males using the Tri Reagent (Molecular Research Center, Inc.) according to the manufacturer’s protocol. Gene specific cDNAs were reverse transcribed and amplified using the Superscript Preamplification System (GIBCOL BRL). A 3’ gene specific primer was used to prime the synthesis of the first strand cDNA. The PCR reactions were performed with a Perkin Elmer 9600 thermal cycler under the following condition: 94°C for 2 minutes for hot start PCR, 30 cycles of 94°C for 1 minute, 60°C for 1 minute and 72°C for 2 minutes, followed by 10 minutes at 72°C. In many cases, the PCR products were a mixture of specific and nonspecific products. The PCR products were shotgun subcloned into the TA cloning vector (Invitrogen) and colony lifts were prepared and screened using probes specific for the cDNA of interest. The positive clones were then sequenced on an automated sequencer (ABI 373). Sequencing multiple alleles of the same gene allowed identification of the polymorphisms specific to the mutagenized chromosomes. For 60AD4 and 60AD8, the mutant cDNAs for these two alleles were under represented (see Chapter 3, Table 3.5), so the genomic region of 60A was sequenced. Briefly, 3 male flies were briefly homogenized with a toothpick in 50 μl of buffer containing 10 mM Tris (PH 8.2), 1 mM EDTA, 25 mM NaCl and 200 μg/ml of proteinase K. The mixture was incubated at 95°C for 15 minutes and after addition of 2.5 μl of 100 mM Pefabloc, for another 15 minutes at 65°C, then spun at 13,000Xg to remove the debris. Ten μl of the supernatant was used for PCR amplification under the same cycling conditions described above. The genomic PCR products were screened and sequenced in the same way as for the cDNAs.
RESULTS

**shn and punt enhance tkv^6 phenotypes**

Prior to conducting the modifier screen, different genetic backgrounds were evaluated. The *tkv^6* mutation was chosen based on its genetic behavior. *tkv^6* is a mutation in a splice acceptor site that results in aberrant in-frame splicing and deletes two amino acids in the extracellular domain of the type I receptor. When expressed in COS1 cells, the mutant receptor fails to bind BMP2 homodimers (Penton *et al.*, 1994). However, *tkv^6* behaves genetically as a hypomorph with phenotypes much weaker than a null allele. It is homozygous viable and the only visible phenotype is thickened wing veins. All other imaginal disc derived structures from *tkv^6* homozygotes appear normal. Interestingly, *tkv^6/Df(2L)tkv2* flies are phenotypically identical to *tkv^6* homozygotes. To test if *tkv^6* was a suitable genetic background for a modifier screen, we examined the effects of lowering the activity of other known *dpp* pathway components. We found that heterozygous mutations in *shn* or *punt* enhanced the *tkv^6* homozygous phenotype. In the *tkv^6* background, *shn^IB* was a dominant enhancer of the venation pattern in the wing and of proximal/distal patterning in the leg. In the wing, longitudinal vein 2 failed to reach the wing margin. In the leg, distal elements such as claws and distal tarsal segments were deleted. Such phenotypes were reminiscent of hypomorphic *dpp* mutant phenotypes (Spencer *et al.*, 1982). *punt^135* was also an enhancer of the *tkv^6* phenotypes. Based on these observations, we reasoned that the *dpp* signaling output through the mutant receptor *tkv^6* was near the threshold for proper patterning of the imaginal discs. It was therefore an appropriate genetic background for identifying new components essential for mediating *dpp* signaling.

**Enhancers of tkv are phenotypically similar to dpp mutants**

The F2 screen for enhancers of *tkv^6* was conducted as outlined in Figure 1. The F2 progeny were screened for enhanced phenotypes in the imaginal disc derived structures. Over 10,000 mutagenized genomes were screened and fourteen dominant enhancers defining seven loci were recovered (Table 1). All of the enhancers were recessive lethal in a wild type background. *tkv^6* homozygotes that are heterozygous for the enhancer mutations display defects in imaginal disc development with varying penetrance. During normal pupal development, the dorsal proximal region of the two wing imaginal discs fuse to form the adult notum. In *tkv^6* flies, the notum appeared normal with a smooth contour and orderly oriented sensory bristles. This pattern was disrupted by the heterozygous enhancer mutations which resulted in a medial cleft in the notum, usually accompanied by abnormally parted bristles on both sides of the cleft. *tkv^6* flies had normally patterned legs. However, a heterozygous *D4* mutation caused deletions of the distal and dorsal structures and occasional duplication of ventrolateral structures such as sex combs on male prothoracic legs. These phenotypes were indistinguishable from
those of dpp\textsuperscript{disk} alleles (Spencer et al., 1982), suggesting that these enhancers act in the dpp signal transduction pathway.

**Enhancer mutations show dosage sensitive interactions with various mutations in the dpp pathway**

Additional evidence that the enhancers mediate dpp signaling came from their dosage sensitive genetic interactions with other known mutations in the dpp pathway, including dpp\textsuperscript{S5}, Df(2L)tkv2, sax\textsuperscript{S}, punt\textsuperscript{135}, Mad,\textsuperscript{P} Med\textsuperscript{4} and shn\textsuperscript{1B} (Table 2). In many cases, the enhancers failed to fully complement these mutations and showed imaginal disc development defects ranging from gaps in wing veins to the notum and leg phenotypes. Since the enhancer mutations are induced in a tkv\textsuperscript{6} background, all enhancer mutations on the second chromosome used in this test also have a tkv\textsuperscript{6} mutation. tkv\textsuperscript{6} alone showed no detectable heterozygous interactions with the dpp pathway mutations examined except for the thickened venation phenotype when it was in trans to tkv\textsuperscript{6} or Df(2R)tkv2, indicating that the phenotypes observed were due to the presence of the enhancer mutations.

To test whether the maternally provided enhancer mutations interfere with early embryonic dpp signaling, we determined whether mutant alleles of the enhancer mutations in the mother would sensitize the zygote to the level of dpp function. The dpp locus is haploinsufficient, indicating that the level of dpp function in the early embryo is critical (Irish and Gelbart, 1987). A series of dpp mutant alleles have been identified that vary in their amount of residual activity (Wharton et al., 1993). One such allele, dpp\textsuperscript{hr27}, normally provides enough residual function to allow viability as a heterozygote. We tested whether mothers heterozygous for dpp\textsuperscript{hr27} and the enhancer mutations would shift the threshold requirement for dpp function, such that dpp\textsuperscript{hr27} would become a dominant lethal in the zygote. In previous studies, certain alleles of tkv, sax, Mad and Med were show to possess this maternal enhancement property (Penton et al., 1994; Nellen et al., 1996; Raftery et al., 1995). Of the enhancer mutations tested, maternally provided D1, D14 and D16 significantly reduced the zygotic viability of dpp\textsuperscript{hr27} (Table 3). However, the lack of enhancement in this test does not necessarily rule out the involvement of the enhancer mutations in the dpp pathway, since in the cases of tkv and sax, dominant negative, rather than loss of function mutations, are required for enhancement (Penton et al., 1994; Nellen et al., 1994).

**Dominant enhancers of tkv\textsuperscript{6}: new alleles of tkv, Mad, Med, punt and 60A**

Meiotic mapping and complementation tests established seven complementation groups for the enhancers. As expected based on the initial evaluation of the tkv\textsuperscript{6} genetic background, new alleles of tkv, Mad, Med and punt were recovered (Table 1). We sequenced the coding regions of the new Mad and punt alleles to determine their molecular lesions (Table 5 and Figure 2). Of the five Mad alleles, three had point mutations in the coding region (Figure 2). Missense
mutations were found in both new *punt* alleles (Figure 2). *tkv*\textsubscript{D17} and *Med*\textsubscript{D5} allelism was based on genetic non-complementation. In addition, a *tkv* transgene was able to rescue the lethality of *tkv*\textsubscript{D17} homozygotes, supporting the conclusion that *D17* is a *tkv* allele (data not shown).

Three of the new *Mad* alleles, *Mad*\textsubscript{D3}, *Mad*\textsubscript{D15} and *Mad*\textsubscript{D24} display dominant female sterility. Embryos derived from heterozygous females had severe patterning defects. The majority of the embryos were partially ventralized. The head structures remained external and the head skeletons were partially deleted. The most posterior structures remained internalized. About 10% of the embryos failed to initiate the germ band retraction process during gastrulation, resulting in a "U" shaped morphology. The dominant female sterility of *Mad*\textsubscript{D3} and *Mad*\textsubscript{D15}, but not of *Mad*\textsubscript{D24}, can be reverted by addition of a *tkv* transgene or a *dpp* transgene maternally (data not shown).

**Preliminary characterizations of the D1 and D2 loci**

Since the *D1* and *D2* complementation groups were only represented by a single ENU allele each, gamma ray screens were carried out to recover more alleles of both loci according to the scheme outlined in Figure 3. No mutations allelic to the *D1* locus were recovered. The mutations, *D2-1*, *D2-2* and *D2-3*, failed to complement *tkv*\textsubscript{D2}. *D2-2* was determined to be most likely allelic to *tkv* since it failed to complement the loss of function alleles of *tkv* (data not shown). *D2-1* and *D2-3* are potentially new alleles of the *D2* locus, or alternatively, alleles of other lethal loci that may be present on the *D2* chromosome. However, *D2-1* and *D2-3* are likely allelic to *D2* because they are lethal over multiple recombinant chromosomes that still retain the ENU-generated *D2* mutation.

To determine the location of *D2* on the chromosome, its lethality over *D2-1* was meiotically mapped and the results are summarized in Table 4. *D2* mapped to meiotic position between 99 and 100 on the right arm of the second chromosome. Consistent with this mapping data, an inversion breakpoint associated with *D2-3* was found to be in the vicinity of this meiotic position through cytological examination (data not shown).

The enhancer mutations were also tested for their ability to suppress a phenotype generated by expressing a constitutively activated *tkv* receptor (*tkvQD*) (Nellen *et al.*, 1996) in a subset of *vestigial* expression domain using the UAS-Gal4 binary system (Brand and Perrimon, 1993). The ectopic expression of *tkvQD* in the *vestigial* domain produced wings that were greatly reduced in size and grossly mispatterned with no discernible venation pattern or margin polarity. Heterozygosity of mutations in *Mad* and *D1* partially restored proximal/distal outgrowth as well as the overall pattern of the wings, whereas heterozygosity for mutations in *dpp* or 60A, which are upstream of *tkv*, failed to suppress the wing phenotype (data not shown). These results are consistent with the role for *Mad* as a
cytoplasmic signal transducer downstream of the tkv receptor, and also suggests that like Mad, the D1 gene may encode a component that functions downstream of the tkv receptor. A heterozygous D2 mutation did not give consistent suppression in this assay (data not shown). The preliminary characterization of the D1 and D2 enhancer mutations suggests that they potentially define loci that were previously not implicated in dpp signaling.

The D4 locus corresponds to the 60A gene, which encodes a BMP-7 homologue

The D4 complementation group was represented by three alleles: D4, D8 and D20, which all dominantly enhanced the imaginal disc phenotypes of tkv^6 homozygotes. Meiotic mapping based on the enhanced phenotypes placed the D4 complementation group in the distal portion of the right arm of the second chromosome (data not shown). Through further deletion mapping, D4 was mapped between 60A2 and 60A3 on the polytene chromosome (Figure 4).

To facilitate the molecular cloning of D4, attempts were made to recover gamma ray alleles of D4. However, nearly 4,000 mutagenized genomes were screened with no recovery of any D4 alleles. Thus, in order to generate transposon insertional mutations in the D4 locus, a P element located closely to the interval where D4 was mapped was mobilized. The P element mutagenesis screen was done as outlined in Figure 5. Three independent P element lines that failed to complement D4 were recovered out of around 4,000 mutagenized genomes. Plasmid rescue experiments were performed to recover the genomic DNA flanking the P element insertion sites (see Materials and Methods). Surprisingly, all P alleles of D4 as well as the parental P element possessed unique genomic regions on one side of the insertion but shared a common region on the opposite side of the insertion. A genomic map of over 50 kb of the D4 region was constructed based on the molecular characterization of four overlapping cosmids in the region (Figure 6). When the P element insertion sites were mapped, it was clear that the P alleles of D4 inserted over a large distance from each other. Based on the observation that they all shared a common flanking genomic sequence on one side, we propose that internal deletion via homologous recombination events occurred between the newly inserted P element and the parental P element. Genomic Southern analysis and the failure to revert the lethality associated with the P insertions by precise excision were consistent with this model.

Southern analysis mapped the 60A transcript to the small deletion interval predicted to disrupt the D4 locus, suggesting that D4 might correspond to the 60A gene. To confirm that the D4 locus corresponds to the 60A gene, the 60A coding regions from D4 alleles were sequenced. All three alleles were found to have nonsense mutations in the prodomain of the 60A polypeptide (Figure 7). Since all three mutations are predicted to eliminate translation of the biologically active mature C-terminal domain, they most likely represent functional nulls of the 60A gene. In fact, sequencing analysis revealed that mutant 60AD4 and 60AD8 mRNAs were greatly under represented in heterozygous animals (Table 5). This could be due to the mRNA instability caused by nonsense mutations (van Hoof and Green,
1996; Kessler and Chasin, 1996; Jenkins et al., 1996). In addition to detecting altered 60A coding regions in D4 alleles, we were also able to fully rescue the lethality associated with the D4 mutations with a 60A transgene (Table 6). These data prove that the D4 locus corresponds to the 60A gene.

**Loss of function phenotypes of 60A**

60A gene product is homologous to mammalian proteins BMP-5 and -7, and was identified initially based on sequence homology (Doctor et al., 1992; Wharton et al., 1991). Its function remained largely unknown due to lack of mutations in this gene. The identification of mutations in the 60A gene allowed analysis of its developmental functions and its role in dpp signaling.

Animals lacking 60A function died at late larval/early pupal stages. One of the striking phenotypes of 60A mutant larvae is the lack of fat body, which gives them a transparent appearance. In roughly 50% of the 60A larvae, the gastric caeca were reduced in length, consistent with the expression of 60A in the gastric caeca (Doctor et al., 1992). These phenotypes are similar to Mad mutant larvae (Sekelsky et al., 1995).

During embryogenesis, 60A is also expressed throughout the visceral mesoderm of the developing midgut (Doctor et al., 1992) suggesting a function for 60A in the development of the embryonic midgut. Indeed, embryos lacking 60A failed to form the first constriction. The homeotic gene Antenapedia (Antp) is expressed in the visceral mesoderm where the first constriction forms and is required for its formation (Reuter and Scott, 1990; Tremml and Bienz, 1989). We examined Antp expression in 60A mutant embryos. Consistent with the lack of the first constriction, Antp protein was greatly reduced or below detectable level. Thus, 60A is required for the formation of the first constriction of the midgut, likely through positively regulating the expression of at least one homeotic gene, Antp in the visceral mesoderm.

**60A maintains an optimal level of dpp signaling in the visceral mesoderm**

The identification of mutations in 60A as dominant enhancers of tkv^6, thus of dpp signaling, in the imaginal discs raised the possibility that 60A is required for optimal signaling by the dpp pathway. To determine if there was a general requirement for 60A in dpp signaling, the effects of 60A mutations on dpp signaling in the visceral mesoderm, where both dpp and 60A are expressed, were examined.

*dpp* is expressed in two distinct domains in the visceral mesoderm (Panganiban et al., 1990b). The anterior expression domain of *dpp* coincides with the gastric caeca primordia, which are immediately anterior to the expression domain of *Sex combs reduced* (Scr) in parasegment (ps) 4. Failure to initiate *dpp* expression in ps3 in *dppshv* mutants results in anterior expansion of *Scr* expression and arrest of the outgrowth of the gastric caeca (Panganiban et al., 1990b; Hursh et al., 1993), indicating a role for *dpp* in repressing *Scr* in ps3. *tkv^6* homozygotes are
homozygous viable, so it is not surprising that all the midgut gene expression patterns examined are essentially normal. Scr expression in tkv^6 or 60A mutant animals was normal. However, in embryos that were double mutant for tkv^6 and 60A, the Scr expression domain extended anteriorly into ps3, also seen in dpp^shv mutants. This suggests that 60A activity is required in ps3 for optimal dpp signaling.

To test whether 60A acts synergistically with dpp elsewhere in the midgut, we examined the gene expression of dpp and Ultrabithorax (Ubx) in ps7 and wingless (wg) in the adjacent ps8. Expression of dpp in ps7 is activated by the homeotic gene Ubx (Immerglück et al., 1990; Panganiban et al., 1990b; Reuter et al., 1990; Capovilla et al., 1994; Sun et al., 1995) and maintained by an autostimulatory circuit involving dpp, Ubx and wg (Hurst et al., 1993; Thüringer et al., 1993b; Thüringer et al., 1993a; Staehling-Hampton and Hoffmann, 1994). Proper expression of all three genes is interdependent in order to coordinate a stably maintained cellular differentiation commitment (Bienz, 1994). The expression of dpp and wg in the visceral mesoderm is required for the induction of the homeotic gene labial (lab) in the underlying endoderm (Immerglück et al., 1990; Panganiban et al., 1990b; Reuter et al., 1990). The absence of dpp function in ps7 disrupts the autoregulatory process and reduces the expression of Ubx, wg and dpp at ps7, leading to the absence of the second constriction in dpp mutant embryos (Immerglück et al., 1990; Panganiban et al., 1990b; Reuter et al., 1990).

We found that animals mutant for either tkv^6 or 60A had normal expression of dpp, wg and Ubx. However, in tkv^660A double mutants, dpp expression in ps3 and ps7 was greatly reduced. The initiation of dpp expression at earlier stages was not affected in the double mutants (not shown), suggesting that the reduction of dpp expression resulted from failure to maintain its expression at later stages. Similarly, in Mad mutants, the initiation of dpp expression in ps3 and ps7 is not affected but the maintenance of dpp expression does not occur (Newfeld et al., 1997). This is because dpp expression is activated directly by Ubx and its maintenance requires positive feedback involving dpp signaling. In tkv^660A mutants, Ubx expression in ps7 and wg expression in ps8 were greatly reduced. The reduction of dpp in ps3 in the double mutants may explain the derepression of Scr observed in these animals.

Ubx is required for repressing Antp in ps6. In Ubx mutants, the Antp domain is extended posteriorly into ps7 (Tremml and Bienz, 1989), indicating a homeotic transformation of ps7 into ps6. A similar phenotype was observed for tkv null embryos (Affolter et al., 1994). Consistent with the argument that absence of 60A function compromises dpp signaling, there was also a posterior expansion of Antp in tkv^660A double mutants. Interestingly, the 60A mutation eliminated the endogenous Antp expression and ectopic Antp was seen in ps7, where Ubx would normally be expressed.
We also examined the expression of lab, the target gene of dpp signaling in the endoderm. Consistent with the gene expression changes in the visceral mesoderm, lab expression was not affected by tkv^ or 60A mutations. However, it was greatly reduced in tkv^60A double mutants. In addition, the gut of the double mutants only formed two-chambers instead of the normal four chambers. This phenotype likely resulted from a failure to form the first constriction due to a lack of 60A function and a failure to form the second constriction due to a lack of dpp signaling. It is unclear why the only constriction observed in the double mutants is positioned somewhat more anterior than a normal third constriction. Taken together with other gene expression changes in the midgut of the tkv^60A double mutants, these data provide further evidence for 60A's role in augmenting the signaling capacity of dpp.

60A enhances the ectodermal phenotypes of tkv^ homozygotes

Previous studies have established dpp's role as a morphogen in patterning the embryonic dorsal epidermis. Higher levels of dpp specify more dorsal cell fates, whereas lower levels of dpp produce more lateral cell fates (Ferguson and Anderson, 1992a; Wharton et al., 1993). Since the potential for dpp to specify cell fates is dosage sensitive, we examined if reducing the dose of 60A influences cell fate specification mediated by dpp signaling.

We compared the cuticle phenotypes of 60A mutants, tkv^ mutants and tkv^60A double mutants. Since tkv^ homozygotes were viable and 60A mutants had no obvious defects until late in development, the cuticular patterns of these mutants were essentially normal. However, tkv^60A homozygotes died as weakly ventralized embryos with head defects and an excessive ventral curvature indicative of defects in germ band retraction and they bear some resemblance to hypomorphic dpp mutants (Wharton et al., 1993). This observation suggested that when dpp signaling is compromised in the embryonic ectoderm, removing 60A activity further attenuated dpp signaling. The weakly ventralized phenotype of the double mutant is consistent with the loss of the highest level of dpp signaling. The relatively mild phenotype may reflect partial rescue by maternally contributed wild type Tkv receptors. Indeed, a quarter of the embryos produced by mothers homozygous for tkv^ and heterozygous for 60A exhibited a dorsal open phenotype similar to that of the zygotic tkv null embryos (Penton et al., 1994).
<table>
<thead>
<tr>
<th>Gene</th>
<th>enhanced phenotype</th>
<th>chromosomal location</th>
<th>number of alleles</th>
<th>allelic to</th>
</tr>
</thead>
<tbody>
<tr>
<td>$D1$</td>
<td>notum</td>
<td>second</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>$D2$</td>
<td>notum</td>
<td>second</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>$D3$</td>
<td>legs and notum</td>
<td>second</td>
<td>5 ($D3, D14, D15, D16, D24$)</td>
<td>$Mad$</td>
</tr>
<tr>
<td>$D17$</td>
<td>legs and notum</td>
<td>second</td>
<td>1</td>
<td>$tkv$</td>
</tr>
<tr>
<td>$D4$</td>
<td>legs and notum</td>
<td>second</td>
<td>3 ($D4, D8, D20$)</td>
<td>$60A$</td>
</tr>
<tr>
<td>$D5$</td>
<td>legs and notum</td>
<td>third</td>
<td>1</td>
<td>$Med$</td>
</tr>
<tr>
<td>$D13$</td>
<td>legs and notum</td>
<td>third</td>
<td>2 ($D13, punt$)</td>
<td>$D18$</td>
</tr>
</tbody>
</table>

The assignment of the enhancer mutations to different genes (complementation groups) is based on genetic mapping and *inter se* complementation tests among enhancer mutations as described in Experimental Methods.
Table 2 Heterozygous interactions between enhancer mutations and *dpp* pathway mutations

<table>
<thead>
<tr>
<th></th>
<th>tkv6</th>
<th>Df(2L) tkv2</th>
<th>dpp85</th>
<th>shnIB</th>
<th>sax5</th>
<th>MadP</th>
<th>Med4</th>
<th>punt</th>
</tr>
</thead>
<tbody>
<tr>
<td>tkv6</td>
<td>-#</td>
<td>-#</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>tkv6D1</td>
<td>67</td>
<td>71</td>
<td>64</td>
<td>60</td>
<td>-</td>
<td>-</td>
<td>31</td>
<td>40</td>
</tr>
<tr>
<td>tkv6D2</td>
<td>43</td>
<td>53</td>
<td>34</td>
<td>-</td>
<td>31</td>
<td>-</td>
<td>-</td>
<td>41</td>
</tr>
<tr>
<td>tkv6D3</td>
<td>94*</td>
<td>100*</td>
<td>96</td>
<td>100*</td>
<td>-</td>
<td>lethal</td>
<td>23</td>
<td>68</td>
</tr>
<tr>
<td>tkv6D15</td>
<td>96*</td>
<td>91*</td>
<td>100</td>
<td>100*</td>
<td>-</td>
<td>lethal</td>
<td>29</td>
<td>78</td>
</tr>
<tr>
<td>tkv6D24</td>
<td>100*</td>
<td>100*</td>
<td>100</td>
<td>100*</td>
<td>-</td>
<td>lethal</td>
<td>41</td>
<td>81</td>
</tr>
<tr>
<td>tkv6D14</td>
<td>98</td>
<td>lethal</td>
<td>68</td>
<td>-</td>
<td>44</td>
<td>lethal</td>
<td>-</td>
<td>35</td>
</tr>
<tr>
<td>tkv6D16</td>
<td>31</td>
<td>50</td>
<td>-</td>
<td>-</td>
<td>41</td>
<td>48</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>tkv6D17</td>
<td>65</td>
<td>lethal</td>
<td>-</td>
<td>15</td>
<td>68</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>tkv6D4</td>
<td>78</td>
<td>83</td>
<td>37</td>
<td>43</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>25</td>
</tr>
<tr>
<td>tkv6D8</td>
<td>82</td>
<td>87</td>
<td>31</td>
<td>39</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>22</td>
</tr>
<tr>
<td>tkv6D20</td>
<td>68</td>
<td>73</td>
<td>25</td>
<td>50</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>31</td>
</tr>
<tr>
<td>D5</td>
<td>47</td>
<td>52</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>lethal</td>
</tr>
<tr>
<td>D13</td>
<td>61</td>
<td>63</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>lethal</td>
</tr>
<tr>
<td>D18</td>
<td>62</td>
<td>67</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>lethal</td>
</tr>
</tbody>
</table>

Numbers represent the percentage of heterozygous progeny with disk phenotypes such as gaps in wing veins, cleft in the notum and/or distal truncation of the legs (lethal, failure to recover the indicated progeny class; asterisk, severe reduction of the indicated progeny class to less than 10% of the expected number; minus, no interactions observed; ND, not done; #, thickened wing veins only, due to non-complementation with *tkv* alleles).
Table 3. Maternal effect of the enhancer mutations on the zygotic viability of *dpp/hr27*

<table>
<thead>
<tr>
<th>Maternal genotype</th>
<th>Wild type wings</th>
<th>Held out wings</th>
<th>Percent of expected</th>
</tr>
</thead>
<tbody>
<tr>
<td>tkv7/dpp/hr27</td>
<td>283</td>
<td>3</td>
<td>3%</td>
</tr>
<tr>
<td>tkv6/dpp/hr27</td>
<td>177</td>
<td>32</td>
<td>54%</td>
</tr>
<tr>
<td>tkv6D1/dpp/hr27</td>
<td>112</td>
<td>3</td>
<td>8%</td>
</tr>
<tr>
<td>tkv6D2/dpp/hr27</td>
<td>200</td>
<td>13</td>
<td>20%</td>
</tr>
<tr>
<td>tkv6D4/dpp/hr27</td>
<td>210</td>
<td>38</td>
<td>54.3%</td>
</tr>
<tr>
<td>tkv6D20/dpp/hr27</td>
<td>238</td>
<td>40</td>
<td>50.4%</td>
</tr>
<tr>
<td>tkv6D14/dpp/hr27</td>
<td>179</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td>tkv6D16/dpp/hr27</td>
<td>87</td>
<td>3</td>
<td>10%</td>
</tr>
<tr>
<td>tkv6D17/dpp/hr27</td>
<td>208</td>
<td>17</td>
<td>24.5%</td>
</tr>
</tbody>
</table>

Females of the genotypes in column one were generated by mating heterozygous *dpp/hr27* females to males heterozygous for the indicated mutations. Females with the indicated maternal genotypes were then mated to males that had a *dpp/d-ho* mutant chromosome (*dppH32 Sp Bl Dp(2;2)dTD48 dpp-d-ho/In(2LR)Gla Bc*). Of the four progeny classes, three had wild type wings. The *dpp/hr27* and the *dpp/d-ho* mutations failed to complement and produced heldout wings. Thus the heldout phenotype was used as an indicator for the zygotic viability of *dpp/hr27*.
Table 4 Meiotic mapping of D2 based on its lethality in trans to D2-1

<table>
<thead>
<tr>
<th>Recombinant classes</th>
<th>Numbers of progeny in the class</th>
</tr>
</thead>
<tbody>
<tr>
<td>pr cn D2</td>
<td>229</td>
</tr>
<tr>
<td>pr cn +</td>
<td>178</td>
</tr>
<tr>
<td>+ + D2</td>
<td>175</td>
</tr>
<tr>
<td>+ + +</td>
<td>238</td>
</tr>
<tr>
<td>pr + D2</td>
<td>4</td>
</tr>
<tr>
<td>pr + +</td>
<td>10</td>
</tr>
<tr>
<td>+ cn D2</td>
<td>14</td>
</tr>
<tr>
<td>+ cn +</td>
<td>7</td>
</tr>
</tbody>
</table>

Total number of recombinants 855

The genetic crosses used to generated these data are described in the Materials and Methods.

Recombinant frequencies (RF) between loci:

- pr cn RF = (4+10+14+7)/855 = 4 (The published distance is 3)
- cn D2 RF = (178+7+175+4)/855 = 42
- pr D2 RF = (178+10+175+14+7x2+4x2)/855 = 46

Based on the published meiotic map locations of pr (54.5) and cn(57.5), the D2 locus was calculated to be between 99 (42+57.5) and 100 (46+54.5). This calculation does not take into consideration the difference in the published and observed value for the distance between pr and cn. Since the distance between the putative D2 locus and pr or cn is very large, the RF can substantially deviate from the actual map distance due to multiple crossing over events. Thus the mapping data presented here should be used with caution.
<table>
<thead>
<tr>
<th>Mutant alleles</th>
<th>Mutant clones/Total clones sequenced</th>
<th>Codon changes</th>
<th>Nature of the mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>MadD3</td>
<td>7/15</td>
<td>No mutations in the coding region</td>
<td></td>
</tr>
<tr>
<td>MadD15</td>
<td>5/14</td>
<td>No mutations in the coding region</td>
<td></td>
</tr>
<tr>
<td>MadD24</td>
<td>7/11</td>
<td>GAC to AAC</td>
<td>G:C to A:T transition</td>
</tr>
<tr>
<td>MadD14</td>
<td>5/11</td>
<td>CAG to TAG</td>
<td>G:C to A:T transition</td>
</tr>
<tr>
<td>MadD16</td>
<td>3/7</td>
<td>TAT to AAT</td>
<td>A:T to T:A transversion</td>
</tr>
<tr>
<td>puntD13</td>
<td>18/28</td>
<td>TGC to TAC</td>
<td>G:C to A:T transition</td>
</tr>
<tr>
<td>puntD18</td>
<td>7/13</td>
<td>GAG to GTG</td>
<td>A:T to T:A transversion</td>
</tr>
<tr>
<td>60AD4</td>
<td>1/14 (cDNA)</td>
<td>One base (C) deletion causes a frame-shift nonsense mutation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4/10 (genomic)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>60AD8</td>
<td>0/12 (cDNA)</td>
<td>TGG to TGA</td>
<td>G:C to A:T transition</td>
</tr>
<tr>
<td></td>
<td>7/14 (genomic)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>60AD20</td>
<td>5/17</td>
<td>CAG to TAG</td>
<td>G:C to A:T transition</td>
</tr>
</tbody>
</table>

See Materials and Methods for the sequence analysis procedures. The mutant clones were identified by comparing chromosome-specific polymorphisms from multiple alleles generated in the same genetic background.
Table 6. Rescue of the lethality of the D4 complementation group by a 60A transgene

<table>
<thead>
<tr>
<th>Number of progeny of the designated genotypes</th>
<th>$p[w^+ 6.5sal]1.2/+$</th>
<th>$+/+$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$D8/tkv^6;pr;cn;D4;(Cy^+)^a$</td>
<td>32 (84%)</td>
<td>0</td>
</tr>
<tr>
<td>$D8;or;tkv^6;pr;cn;D4/CyO;a$</td>
<td>76</td>
<td>78</td>
</tr>
<tr>
<td>$D20/tkv^6;pr;cn;D4;(Cy^+)^b$</td>
<td>69 (&gt;100%)</td>
<td>0</td>
</tr>
<tr>
<td>$D20;or;tkv^6;pr;cn;D4/CyO;b$</td>
<td>83</td>
<td>91</td>
</tr>
<tr>
<td>$Df((2R)bw^{Dra}/tkv^6;pr;cn;D4;(Cy^+)c$</td>
<td>37 (98%)</td>
<td>0</td>
</tr>
<tr>
<td>$Df(2R)bw^{Dra};or;tkv^6;pr;cn;D4/CyOc$</td>
<td>75</td>
<td>90</td>
</tr>
</tbody>
</table>

$p[w^+ 6.5sal]1.2$ is a transgene carrying 6.5 kb of genomic DNA containing 60A as well as two other coding sequences, peter pan and sahara (Wharton, K. personal communication). D4, D8 and D20 fully complemented a small deficiency ($Dr(2R)P10-107$) that deletes peter pan and sahara (data not shown), indicating that they are not allelic to either peter pan or sahara.

The progeny derive from the following crosses:

- $tkv^6\;pr\;cn\;D4/CyO;\;p[w^+ 6.5sal]1.2/+\;X\;D8/CyO;\;+/+$
- $tkv^6\;pr\;cn\;D4/CyO;\;p[w^+ 6.5sal]1.2/+\;X\;D20/CyO;\;+/+$
- $tkv^6\;pr\;cn\;D4/CyO;\;p[w^+ 6.5sal]1.2/+\;X\;Df(2R)bw^{Dra}/CyO;\;+/+$

The first column and row describe the genotypes of the second chromosome and third chromosome, respectively. The number in parentheses are the percentages of expected $Cy^+$ progeny rescued to adulthood by one copy of the transgene.
Figure 1. Scheme for the F2 enhancer screen of tkv$^6$.
ENU, Ethylnitrosourea; T(2;3); a translocation between CyO chromosome and TM6,B chromosome which forces the cosegregation of the second and third chromosome. Asterisks indicate mutagenized chromosomes. See Experimental Methods for detailed description of full genotypes and the procedure.

1. For modifiers on the second chromosome

\[
\frac{tkv^6 \text{ pr} \text{ cn}^*}{\text{CyO, Cy pr cn}} ; + \quad \frac{+}{\text{T(2;3)}} \quad \frac{tkv^6}{\text{CyO, Cy pr cn}} ; + \quad \frac{\text{TM2, Ubx e}}{\text{TM6, B e Tb}} \quad \frac{\text{♀♀}}{} \]

retest for modification and make balanced stock

2. For modifiers on the third chromosome

\[
\frac{tkv^6 \text{ pr} \text{ cn}}{\text{CyO, Cy pr cn}} ; +^* \quad \frac{+^*}{\text{TM2 or TM6, B}} \quad \frac{tkv^6}{\text{CyO, Cy pr cn}} ; +^* \quad \frac{\text{TM2, Ubx e}}{\text{TM6, B e Tb}} \quad \frac{♀♀}{♀♀} \]

retest for modification and make balanced stock
Figure 2. Molecular lesions in the new alleles of *Mad* and *punt*.
The position and the amino acid changes of each mutation are indicated below the schematic diagram of the protein. Asterisks represent nonsense mutations. (A) Mutations in new *Mad* alleles. Closed bars represent the highly conserved Mad Homology domain 1 and 2 (MH1 and MH2). The hatched box at the Carboxy terminal represents the mutation hot spot. (B) Mutations in new *punt* alleles. The structural features of the *punt* Type II receptor shown are the putative signal peptide (oval box), the extracellular cysteine residues (vertical bars), the transmembrane domain (hatched box) and the kinase domain (open box).

A. MAD

\[ \text{NH}_2 \rightarrow \text{MH1} \rightarrow \text{MH2} \rightarrow \text{COOH} \]

- \( \text{Mad}^{D14}Q168^* \)
- \( \text{Mad}^{D16}Y255N \)
- \( \text{Mad}^{D24}D437N \)

B. PUNT

\[ \text{punt}^{D13}C67Y \rightarrow \text{punt}^{D18}E378V \]
Figure 3. Scheme for the gamma ray screen for D2 alleles.
The asterisk labels the chromosomes exposed to the gamma ray irradiation. See Experimental Methods for a more detailed description of the procedure.

4,500 rad γ ray

\[ +/+ \quad \delta \sigma \quad \times \quad \text{In}(2LR) \text{Gla Bc/CyO} \quad \text{♀♀} \]

\[ \downarrow \]

\[ +^*/\text{CyO} \quad \delta \sigma \quad \times \quad \text{tkv}^6 \text{ pr cn D2/CyO} \quad \text{♀♀} \]

\[ \downarrow \quad \text{screen for the absence of Cy}^+ \text{ progeny} \]

\[ +^*/\text{CyO} \quad \delta \sigma \quad \times \quad \text{tkv}^6 \text{ pr cn D2/CyO} \quad \text{♀♀} \]

\[ \downarrow \quad \text{retest and make balanced stocks} \]

\[ D2-1 \]
\[ D2-2 \]
\[ D2-3 \]
Figure 4 Deletion mapping of the D4 locus.
Shown is the polytene chromosome region of 60A. Numbers above the polytene chromosome represent the subdivisions within 60A. Deficiency stocks used for the mapping are listed below the polytene map with the names and the regions deleted indicated. Open or hatched boxes represent the complementing or non-complementing deficiencies respectively. The region between the vertical dashed lines represent the interval to which D4 was mapped.

[Diagram showing complementing and non-complementing deficiencies with regions labeled]

Complementing deficiency

Non-complementing deficiency

<table>
<thead>
<tr>
<th>Complementing deficiency</th>
<th>Non-complementing deficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Df(2R)OV1 59F5-6; 60A1</td>
<td>Df(2R)b23 59F8; 60A3</td>
</tr>
<tr>
<td>Df(2R)X32 59F4; 60A2</td>
<td>Df(2R)bwDra 59D3-4; 60A4-5</td>
</tr>
<tr>
<td></td>
<td>Df(2R)bwS46 59D8-11; 60A7</td>
</tr>
</tbody>
</table>
Figure 5. Scheme for the P element mutagenesis of $D4$. 
$\Delta 2-3$ represents the source of the transposase used in the screen. The details of the procedure of the mutagenesis are described in Experimental Methods.

\[ \begin{align*}
  w; p[w^+](2)k/CyO; +/+ \quad \text{♀♀} \quad & \times \quad w; Sp/CyO; Sb \Delta 2-3/+ \quad \text{♂♂} \\
  \downarrow \\
  w; p[w^+](2)k/CyO; Sb \Delta 2-3/+ \quad \text{♂♂} \quad & \times \quad w; In(2LR)Gla Bc/CyO; TM2/TM6,B \quad \text{♀♀} \\
  \downarrow \\
  4,000 \left( w; p[w^+]/CyO; +/TM2 \text{ or } 6,B \quad \text{♂} \right) \quad & \times \quad W; tkv^6 D4/CyO; TM2/TM6B \quad \text{♀♀} \\
  \downarrow \\
  \text{screen for the absence of } Cy^+ \text{ progeny} \\
  w; D4p[w^+]/CyO \quad \text{♂♂} \quad & \times \quad W; tkv^6 D4/CyO; TM2/TM6,B \quad \text{♀♀} \\
  \downarrow \\
  \text{retest and make balanced stock} \\
  w; D4p[w^+]/CyO \text{ stock (} D4p^1, D4p^2, D4p^3 \text{)}
\end{align*} \]
Figure 6. The genomic organization of the D4 locus.
The genomic map of the D4 region is shown with XbaI (X) and NotI (N) restriction enzyme sites indicated. The closed triangle represents the parental P element, l(2)k, of which the new P alleles of D4 (open triangles) are derived. The hatched boxes represent the genomic fragment rescued from the left side of the insertion using the EcoRI restriction enzyme. The closed box represents the genomic fragment rescued from the right side of the insertion using the BglII restriction enzyme. Southern and sequence analysis indicated that the BglII rescued fragment from all the P alleles of D4 were identical to that of the parental P element. The overlapping cosmids isolated using the EcoRI rescued fragment of D4-P2 are shown below the genomic map. The 60A transcript is indicated with a bracket.
Figure 7. Mutations in D4 alter the 60A coding region.
Shown is a schematic representation of the 60A polypeptide with the structural features indicated. The seven Cs within the mature domain represent the seven conserved cysteines. The arrows point to the position of the mutations in the coding region. The molecular lesions are indicated. 60AD8 and 60AD20 are nonsense mutations resulting from single nucleotide substitutions. 60AD4 is a frame-shift nonsense mutation resulting from a single nucleotide deletion.

60A

<table>
<thead>
<tr>
<th>Leader</th>
<th>Prodomain</th>
<th>Mature domain</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH2</td>
<td></td>
<td>C CC CC CC CC COOH</td>
</tr>
</tbody>
</table>

a. 60AD20 Q137*
b. 60AD8 W179*
c. 60AD4 V237* (frame shift nonsense mutation)
DISCUSSION

The haploinsufficiency of the dpp locus reflects the sensitivity of developmental processes to a reduction in dpp signaling. We have carried out a genetic screen to search for modifiers of tkv<sup>6</sup>, a hypomorphic allele of the tkv gene, which encodes a type I receptor for dpp. tkv has been implicated in all aspects of dpp signaling both in vitro and in vivo (Penton et al., 1994; Nellen et al., 1994; Brummel et al., 1994; Affolter et al., 1994; Burke and Basler, 1996a; de Celis, 1997; Singer et al., 1997). Thus the modifiers of tkv<sup>6</sup> are likely to be integral components of the dpp signal transduction pathway. In fact, new alleles of several genes known to mediate dpp signaling were recovered. These included tkv, punt, Mad and Med. These mutations dominantly enhanced the mild imaginal disc phenotypes of tkv<sup>6</sup>. Recovery of these mutations validated the specificity of the screen.

Five new alleles of Mad, a key signal transducer in dpp signaling (Newfeld et al., 1996; Kim et al., 1997) were isolated. Three have point mutations in the coding region (Figure 3.4A). The molecular lesions correlate with their phenotypic properties. Mad<sup>D14</sup> has a nonsense mutation predicted to produce a truncated protein with only the conserved MH1 domain and it behaves genetically as a null. Mad<sup>D16</sup> has a tyrosine to asparagine change in the divergent linker region and behaves genetically as a hypomorph, suggesting that the amino acid change only partially affects the protein function. The MH2 domains of Mad-related proteins (Smads) are highly conserved. The three dimensional structure of the MH2 domain of Smad4 indicates that it mediates the homotrimerization of Smad proteins. The intact conformation of the homotrimer is essential for the assembly of a heterohexamer with another Smad homotrimer in response to receptor activation (Hata et al., 1997; Shi et al., 1997). Many of the Smad mutations associated with tumors or developmental defects map within the MH2 domain. Based on the crystal structure of the carboxy terminal domain of Smad4 (Shi et al., 1997), the invariant aspartic acid mutated to asparagine in Mad<sup>D24</sup> maps to the trimer interface region, which is critical for trimerization. The corresponding residue in Smad2 is mutated in colon cancers (Eppert et al., 1996). Interestingly, the Mad<sup>D24</sup> mutation resulted in dominant female sterility, which is not observed with Mad null alleles. This suggests that the Mad<sup>D24</sup> mutation has a dominant negative effect, perhaps by forming inactive oligomers with the wild type protein in the heterozygotes.

Analysis of the two new punt alleles also provides evidence for the in vivo importance of conserved structural motifs in this type II dpp receptor. punt<sup>D13</sup> has a cysteine to a tyrosine change in the extracellular cysteine cluster characteristic of all receptors for TGF-β superfamily members (Massagué et al., 1994). punt<sup>D18</sup> changes the highly conserved glutamic acid to a valine in the catalytic core of the kinase domain (Hanks et al., 1988), where another punt mutation, punt<sup>135</sup> has been
mapped (Ruberte et al., 1995). Like punt\textsuperscript{135}, both new punt alleles display some
temperature sensitivity, suggesting that they are not protein nulls. To date, no null
mutations in the punt locus have been reported, suggesting that like dpp, punt may
be haploinsufficient.

Besides providing information about the structure-function relationship, the
sequencing data also added useful information on the germline mutation spectrum
of ENU. Of eight ENU alleles sequenced that had mutations in the respective
coding regions, five were GC to AT transitions, two were AT to TA transversions
and one involved a single nucleotide deletion. The frequencies of the two types of
nucleotide substitution is comparable to the reported frequencies of the ENU
generated mutations in Drosophila (see references in Marker et al., 1997). Although
ENU generated deletions were not previously reported in Drosophila, they were
recovered at a low frequency in E. coli and mice (see references in Marker et al.,
1997).

No new alleles of shn were isolated although it acted as an enhancer of tkv\textsuperscript{6}
in the initial test. The enhancement by shn\textsuperscript{1B} may be allele specific, such that a
particular form of Shn mutant protein is needed to produce an enhancement effect.
Consistent with this, shn\textsuperscript{P}, which makes no detectable protein (Staehling-Hampton
et al., 1995), failed to enhance the tkv\textsuperscript{6} phenotype (data not shown). No dpp alleles
were recovered either, possibly due to the haploinsufficiency associated with the
locus and the fact that most hypomorphic dpp mutations affect the regulatory
regions of dpp, which are less likely to be affected by chemical mutagens such as
ENU.

Preliminary characterization of D1 and D2 indicates that they potentially
define two new loci. Both mutations have extensive genetic interactions with other
components of the dpp pathway. Heterozygous interactions between recessive
mutations are rare and are usually observed for genes that encode proteins involved
in the same pathway and sometimes may reflect a physical association between the
proteins. Thus the heterozygous interactions between these two enhancers and the
dpp pathway mutations argues for their role(s) in the dpp pathway. In addition, the
D1 mutation maternally enhanced the zygotic lethality of dpp\textsuperscript{27}. The suppression of
a phenotype associated with an activated tkv\textsuperscript{QD} by heterozygous D1 mutation
suggests that it may act downstream of tkv. However, the epistatic relationships
inferred from this experiment should be taken with caution because it not known
whether the tkv\textsuperscript{QD} receptor is fully activated or not. If it is not fully activated, then
it would be possible to achieve suppression by a quantitative reduction in the
activity of an upstream component. More thorough characterizations of these two
loci are in progress to determine their molecular identity and role(s) in the dpp
pathway.
Mutations in the 60A gene, which encodes the Drosophila homologue of mammalian BMP-5 and -7 (Doctor et al., 1992; Wharton et al., 1991) were identified in a modifier screen for components involved in dpp pathway. The fact that in a screen of the entire genome, 60A mutations were recovered multiple times as dominant enhancers of a mutant dpp receptor provides strong evidence for its involvement in dpp signaling.

Phenotypic analysis of 60A single mutants and tkv^60A double mutants revealed both dpp-independent and dpp-dependent functions for 60A. 60A protein is expressed in a broad pattern throughout development, but is enriched in the developing gut region (Doctor et al., 1992), suggesting a role for 60A in gut morphogenesis. 60A mutants lack the first constriction of the embryonic midgut and Antp expression in ps6. This suggests that 60A is required for the formation of the first constriction, possibly through regulating Antp expression. This function is independent of dpp signaling, since mutations in dpp and its receptors only disrupt the formation of the second constriction. Neither tkv, sax nor punt affects the formation of the first constriction, suggesting that there is either redundancy or that a different receptor system may be responsible for mediating 60A signaling to pattern the first constriction. It would be interesting to see if Atri (Childs et al., 1993), a type I receptor and STK-D (Ruberte et al., 1995), a type II receptor in Drosophila, both of unknown function, are mediators of 60A signaling at the site of the first constriction.

The role of 60A in potentiating dpp signaling in revealed by the fact that 60A mutations are dominant enhancers of a sensitized dpp pathway. This is most obvious in the visceral mesoderm of the midgut where dpp signaling is required to regulate homeotic gene expression and to positively stimulate its own expression through an autocrine mechanism. Although dpp signaling in the visceral mesoderm appears intact in 60A mutants, a requirement for 60A is revealed in the tkv^60A double mutants. These double mutants show derepression of Scr in the anterior midgut and the loss of expression of the dpp target genes, wg, Ubx and dpp, in the visceral mesoderm and lab in the underlying endoderm, consistent with the loss of positive feedback on dpp. A similar requirement for 60A is observed in the dorsal/ventral patterning of the embryonic ectoderm. The enhanced phenotypes of the adult appendages closely resemble those of dpp hypomorphic mutations (Spencer et al., 1982), suggesting that 60A activity is also required in imaginal disc patterning. It is interesting that the imaginal discs are more sensitive to reduction of 60A function, as a 50% reduction in 60A function is sufficient to produce a phenotype in a tkv^6 genetic background. This may reflect a differential threshold requirement for dpp signaling in different tissues. Taken together, our data argue for an involvement of 60A in dpp signaling at different developmental stages and in various tissues.

In a signaling system with multiple interacting ligands, the interpretation of any single mutant phenotypes must consider the effect of losing both homomeric
and possible heteromeric ligands. Therefore, the functions of the dpp pathway may well be a composite input from Dpp homodimers, Dpp/Scw and Dpp/60A heterodimers. An alternative model is that 60A homodimers function in an additive fashion with Dpp homodimers at sites of overlapping expression. However, the loss of function phenotypes of dpp are as severe as the loss of function phenotypes of its downstream components, such as tkv or Mad (Padgett et al., 1987; Penton et al., 1994; Nellen et al., 1994; Newfeld et al., 1996; Newfeld et al., 1997), suggesting that there is very little requirement for 60A signaling, if any at all, from 60A homodimers in dpp-dependent events. Therefore, we believe it is unlikely that 60A homodimers play a significant role in dpp dependent processes. Rather, we favor the interpretation that Dpp/60A heterodimers form at sites of overlapping expression and participate with Dpp homodimers in multiple signaling events.

The overlapping expression patterns of multiple murine BMPs have led to the suggestion that they may act combinatorially during development (Lyons et al., 1995). Given the dimeric nature of the TGF-β superfamily ligands, one mechanism to achieve such a combinatorial effect is to form functional heterodimers. Heterodimers of Xenopus BMP-4 and -7 have been generated in vitro (Hazama et al., 1995) and have been shown to be more potent in bone (Aono et al., 1995) and mesoderm inducing assays (Suzuki et al., 1997) than either homodimer. In Drosophila, another BMP-related factor, Scw, is proposed to up-regulate the activity of Dpp by forming Dpp/Scw heterodimers in the dorsal/ventral patterning of the embryonic ectoderm (Arora et al., 1994). The broad distribution of 60A proteins provides an opportunity for forming Dpp/60A heterodimers. Unlike scw null mutations, no obvious disruption of dpp signaling is observed in 60A null mutants, suggesting that Dpp/60A heterodimers are not as limiting as Dpp/Scw heterodimers, but partially redundant with Dpp homodimers. The first constriction phenotype of 60A mutants is unique, suggesting that it may be a function of 60A homodimers.

The tkv^6 receptor failed to bind homomeric ligands when expressed in COS cells (Penton et al., 1994), although dpp signaling is largely intact in tkv^6 mutants. Thus, it would be interesting to determine if the mutant receptor still binds Dpp/60A and/or Dpp/scw heterodimers. If the tkv^6 mutation specifically reduces homodimer binding but had little or no effect on heterodimer binding, it would indicate that Dpp/60A heterodimers are capable of providing sufficient signaling in the absence of signaling from Dpp homodimers. The severe midgut phenotypes of tkv^6/60A double mutants could be due to additional loss of signaling from Dpp/60A heterodimers. This would be consistent with our hypothesis that 60A potentiates dpp signaling by forming heterodimers with Dpp. The relatively mild cuticular defects of tkv^6/60A double mutants lacking maternally provided wild type Tkv receptors may be due to residual Dpp/Scw heterodimer signaling through the mutant tkv^6 receptor during early embryogenesis.
CONCLUSIONS

The genetic approach supported by the grant worked. New mutations were recovered in virtually every known component of the dpp signaling pathway. The importance of another ligand, the BMP7 homolog 60A, in dpp signaling was discovered for the first time. A primary goal was to discover new components and to use the Drosophila genes as probes to identify the human genes and study their role, if any, in human breast cancer. D1 and D2 are tkv\textsuperscript{6} enhancer mutations that potentially represent two novel loci. They interact extensively with other mutations in the dpp pathway. D1, in particular, is able to partially suppress a dominant phenotype generated by expressing a constitutively active tkv receptor in the wing, suggesting that it may act downstream of the receptor. On-going experiments indicate that D1 may be a mutation in dpp itself or in a very closely linked gene. The focus of on-going efforts has shifted to D2. We want to know what kind of protein D2 encodes and to understand its role in dpp signal transduction. The location of D2 has been determined by meiotic and deletion mapping. Pre-existing mutations have been tested for possible allelism but no other alleles were found. One other allele was obtained from the laboratory of Dr. Richard Padgett, Rutgers University. It was recovered there independently by a different modifier strategy. DNA from the region was obtained as a cosmid contig from the European Drosophila Genome Project. Current experiments are seeking to identify which transcript is altered by the D2 mutations.

It is likely that there are additional factors important for the regulation of the dpp pathway that were not uncovered by the modifier screen accomplished in this grant period. For example, there may be a cytoplasmic sequestering factor regulating the nuclear translocation of Mad proteins and potential transcriptional partner(s) of Mad, like FAST-1 in Xenopus (Chen et al., 1996). In addition to the general signal transducers, there may be tissue specific effectors of the dpp pathway. Since the tkv\textsuperscript{6} background has been proven to be a low-noise background and only 10,000 mutagenized genomes have been screened, it is possible that scaling up the screen, would unveil more modifier loci. Also, the dominant female sterility associated with some Mad alleles recovered in the screen reported here may be a useful genetic background to screen for mutations that would revert the dominant female sterility. Finally, one can look for suppressors of the wing phenotypes caused by expressing the constitutively active tkv in the vestigial pattern. Possible suppressors of this phenotype may be loss of function mutations in the positive regulators of the dpp pathway or activating mutations in the factors that negatively regulate the pathway.

The remarkably diverse biological activities elicited by TGF-\(\beta\)-related factors confers clinical potential for treating cancer, stimulating tissue repair and modulating development. It is very satisfying to realize that what we have learned in this tiny red-eyed creature has had and will continue to have a profound impact on our understanding of similar biological processes present in humans.
REFERENCES


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A genetic screen for modifiers of Drosophila decapentaplegic signaling identifies mutations in punt, Mothers against dpp and the BMP-7 homologue, 60A

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SUMMARY

decapentaplegic (dpp) is a Transforming Growth Factor beta (TGF-ß)-related growth factor that controls multiple developmental processes in Drosophila. To identify components involved in dpp signaling, we carried out a genetic screen for dominant enhancer mutations of a hypomorphic allele of thick veins (tkv), a type I receptor for dpp. We recovered new alleles of tkv, punt, Mothers against dpp (Mad) and Medea (Med), all of which are known to mediate dpp signaling. We also recovered mutations in the 60A gene which encodes another TGF-ß-related factor in Drosophila. DNA sequence analysis established that all three 60A alleles were nonsense mutations in the prodomain of the 60A polypeptide. These mutations in 60A caused defects in midgut morphogenesis and fat body differentiation. We present evidence that when dpp signaling is compromised, lowering the level of 60A impairs several dpp-dependent developmental processes examined, including the patterning of the visceral mesoderm, the embryonic ectoderm and the imaginal discs. These results provide the first in vivo evidence for the involvement of 60A in the dpp pathway. We propose that 60A activity is required to maintain optimal signaling capacity of the dpp pathway, possibly by forming biologically active heterodimers with Dpp proteins.

Key words: dpp, punt, Mad, 60A, Modifier screen, TGF-ß, Drosophila

INTRODUCTION

The Transforming Growth Factor-beta (TGF-ß) superfamily is a family of conserved polypeptide growth factors that regulate diverse biological activities (reviewed by Kingsley, 1994a; Massagué, 1996). In particular, within the TGF-ß superfamily, the Bone Morphogenetic Proteins (BMPs) are critical regulators of cell proliferation, cell death, cell fate specification and organogenesis (reviewed by Kingsley, 1994b; Hogan, 1996). The versatile signaling capacity of TGF-ß-related factors partially stems from extensive post-translational modifications that occur during the maturation process. These factors are synthesized as large precursor molecules, which undergo homomeric or heteromeric dimerization and subsequent proteolytic cleavage to yield the bioactive carboxy terminal portion (Roberts and Sporn, 1990). Upon secretion, their association with extracellular binding proteins is important for regulating their access to cell surface receptors (reviewed by Miyazono et al., 1993). The interaction with a heteromeric transmembrane receptor system composed of two distinct serine/threonine kinases (type I and II receptors) adds one more level of complexity to TGF-ß-related signaling (Massagué and Weis-Garcia, 1996). In order to understand the complex processes involved

in TGF-ß signaling, we focus on decapentaplegic (dpp), a Drosophila gene that is functionally interchangeable with mammalian BMP-2 and BMP-4 (Padgett et al., 1993; Sampath et al., 1993).

dpp has a dynamic expression pattern and multiple functions throughout Drosophila development. At the blastoderm stage, dpp transcripts are restricted dorsally (St. Johnston and Gelbart, 1987) where it functions as a morphogen to specify distinct dorsal structures (Ferguson and Anderson, 1992a,b; Wharton et al., 1993). Later during embryogenesis, dpp is expressed in the ectoderm (Jackson and Hoffmann, 1994), where it induces dorsal mesoderm differentiation (Stachling-Hampton et al., 1994). In the visceral mesoderm, dpp is expressed in discrete domains (Panganiban et al., 1990b) to regulate the expression of several homeotic genes in different tissue layers (Bienz, 1994). dpp is also expressed in specific positions in the larval imaginal discs (Masucli et al., 1990) to control the proliferation and patterning of adult appendages (reviewed by Brook et al., 1996).

In addition to dpp, 60A and scw are two other BMP-related genes in Drosophila (Arora et al., 1994; Doctor et al., 1992; Wharton et al., 1991). Sow proteins have been proposed to enhance dpp activity during early embryogenesis by forming heterodimers with Dpp (Arora et al., 1994). The role of 60A in
development was unclear due to the lack of knowledge of the phenotypic consequences of disrupting 60A function.

In Drosophila, two type I receptors encoded by saxophone (sax) and thick veins (tkv) and one type II receptor encoded by punt have been shown to be functional dpp receptors (Penton et al., 1994; Brummel et al., 1994; Nellen et al., 1994; Ruberte et al., 1995; Letsou et al., 1995; Xie et al., 1994). Mothers against dpp (Mad), Medea (Med) and schnuurri (shn) were identified through genetic interactions with dpp (Raftery et al., 1995; Sekelsky et al., 1995; Staehling-Hampton et al., 1995; Grieder et al., 1995; Arora et al., 1995). shn encodes a protein related to human zinc finger transcription factor PRDII/MBPI/HIV-EP1 (Staehling-Hampton et al., 1995; Arora et al., 1995; Grieder et al., 1995). Mad-related proteins (Smads) have been isolated from a wide range of distantly related organisms. Genetic and biochemical evidence indicated that Smads are key signal transducers, linking events between receptor activation and changes in target gene expression (Derynck and Zhang, 1996; Massagué, 1996; Wrana and Pawson, 1997).

The dosage-sensitive nature of dpp signaling prompted us to use modifier genetics to identify additional components in the dpp pathway. This approach exploits synergistic interactions between components in the same biological pathway. We sensitized the dpp pathway using a hypomorphic dpp receptor, tkv<sup><i>b</i></sup>, which had a mild visible phenotype. We reasoned that, in this genetic background where dpp signaling is below optimal level, a two-fold reduction in the activities of other signaling components in the pathway would produce a modified phenotype. So we screened for mutations that dominantly modified tkv<sup><i>b</i></sup> phenotype. Such an approach has successfully identified components in several Drosophila signal transduction pathways, including the sevenless receptor tyrosine kinase pathway (Simon et al., 1991), the Abelson cytoplasmic tyrosine kinase pathway (Gertler et al., 1990) and the dpp pathway (Raftery et al., 1995).

In our screen, we recovered new alleles of tkv, punt, Mad and Med; all are known to mediate dpp signaling. Two other complementation groups were identified that potentially represent new components in the pathway. Most significantly, mutations in 60A, the Drosophila homologue of BMP-7, were recovered as enhancers for the sensitized dpp pathway. We describe the loss-of-function phenotypes of 60A and present the first in vivo evidence that 60A acts synergistically with dpp in several developmental processes.

**MATERIALS AND METHODS**

**Drosophila stocks**

Drosophila stocks were cultured on standard cornmeal yeast extract sugar medium at 25°C. Canton S. was used as the wild-type stock. sax<sup>3</sup> was described in Twombly et al. (1996). Mad<sup>3</sup> is described in Sekelsky et al. (1995). Med<sup>3</sup> was described in Raftery et al. (1995). All other mutants and chromosomes are referenced in Flybase (http://cbbridges.harvard.edu).

**Isolation and analysis of enhancer mutations of tkv<sup><i>b</i></sup>**

The initial attempts to recover modifiers of tkv<sup><i>b</i></sup> in an F<sub>2</sub> screen was not successful because of greatly reduced viability and fertility of the flies with enhanced phenotypes. Thus an F<sub>3</sub> screen was carried out. pr cn was recombined onto the tkv<sup><i>b</i></sup> chromosome and the stock was made isogenic for the second and the third chromosomes. tkv<sup><i>b</i></sup> pr cn/CyO males were mutagenized with 3.4 M ethylnitrosourea (ENU) and mated to females with a translocation between CyO and TM6.B to force the co-segregation of the second and third chromosome. Individual male progeny were mated to tkv<sup><i>b</i></sup>/CyO; TM2/TM6.B females. Enhancement of the phenotypes of the imaginal-disc-derived structures were screened in the progeny homozygous for tkv<sup><i>b</i></sup>. The enhancer mutations were recovered from the siblings of the enhanced progeny and crossed to tkv<sup><i>b</i></sup>/CyO; TM2/TM6.B females for retesting and to establish balanced stocks.

The number of complementation groups was determined from inter se crosses among enhancers. For enhancers on the second chromosome, a tkv<sup><i>b</i></sup> transgene on the third chromosome (Y. C. and F. M. H., unpublished data) was used to compensate for the tkv<sup><i>b</i></sup> mutation present on the second chromosome. Allelism with known mutations was established by genetic non-complementation and by meiotic and deletion mapping. The Sp Bl<sup>Lm<sub>ts</sub></sup> Be Pu<sup>d</sup> Pwt<sup>b</sup> chromosome was used for meiotically mapping enhancers on the second chromosome.

**Sequencing of mutant alleles**

Total RNAs were isolated from heterozygous Mad and punt females and heterozygous 60A males using the Tri Reagent (Molecular Research Center, Inc.). Gene-specific cDNAs were reverse transcribed and amplified using the Superscript Preamplification System (GIBCO BRL). The PCR products were subcloned into TA cloning vectors (Invitrogen) and sequenced on an automated sequencer (ABI 373). Sequencing multiple alleles of the same gene allowed identification of the polymorphisms specific to the mutagenized chromosomes. For 60A<sup>D1</sup> and 60A<sup>D8</sup>, the genomic region of 60A was sequenced in a similar fashion because the mutant cDNAs were under-represented.

**Preparation of larval gut, cuticle and adult appendages**

Larval gut was dissected and mounted according to Masucci and Hoffmann (1993). Cuticles were prepared as described previously (Struhl, 1989). Wings and legs were mounted in Gary's magic mounting media (Ashburner, 1989).

**Antibody staining and identification of mutant embryos**

Anti-Scr, Anti-Ubx, Anti-Lab antibodies were gifts of Dr Matthew Scott, Stanford University. Anti-Wg antibody was a gift of Dr Roel Nusse, Stanford University. Anti-Dpp antibody was described in Panganiban et al. (1990a). Antibody stainings were done as previously described (Panganiban et al., 1990b; Staehling-Hampton and Hoffmann, 1994; Reuter et al., 1990). All stocks used for antibody staining were balanced over a CyO chromosome with an elav-lacZ enhancer trap to allow unambiguous identification of mutant embryos.

**RESULTS**

**shn and punt enhance tkv<sup><i>b</i></sup> phenotypes**

tkv<sup><i>b</i></sup> is a mutation in a splice acceptor site that results in aberrant in-frame splicing, deleting two extracellular amino acids of the receptor. When expressed in COS1 cells, the mutant receptor fails to bind BMP-2 homodimers (Penton et al., 1994). However, tkv<sup><i>b</i></sup> behaves genetically as a hypomorph. In contrast to the embryonic lethal tkv null alleles, tkv<sup><i>b</i></sup> is homozygous viable and the only visible phenotype is the thickened wing veins (Fig. 1B). All other imaginal-disc-derived structures of tkv<sup><i>b</i></sup> homozygotes appear normal (Figs 1C, 3A,C). Interestingly, tkv<sup><i>b</i></sup>/Df(2L)tkv<sup>2</sup> flies are phenotypically identical to tkv<sup><i>b</i></sup> homozygotes (data not shown). To test if tkv<sup><i>b</i></sup> is a suitable genetic background for a modifier screen, we examined the effects of lowering the activity of other known dpp pathway components. We found that
Fig. 1. shn enhanced tkv\textsuperscript{6} homozygous phenotypes. (A) Wild-type wing. (B) tkv\textsuperscript{6} wing. Thickening of the cross veins and the terminals of the longitudinal veins were evident (arrowhead). (C) Phenotypically normal tkv\textsuperscript{6} wing. Thickening of the cross veins and the terminals of the longitudinal veins were evident (arrowhead). (D) tkv\textsuperscript{6}/tkv\textsuperscript{6} truncated (arrowhead). (E) Phenotypically normal tkv\textsuperscript{6}/tkv\textsuperscript{6} wing. Besides the thickened wing veins, the longitudinal vein 2 was near the wing margin (Fig. 3B). In the wing, longitudinal vein 2 failed to reach the wing margin (Fig. 1E). In the leg, distal elements such as claws and distal tarsal segments were deleted (Fig. 1E). Such phenotypes were reminiscent of hypomorphic dpp phenotypes (Spencer et al., 1982). punt\textsuperscript{135} also enhanced the tkv\textsuperscript{6} phenotypes (data not shown). Based on these observations, we reasoned that the dpp signaling output through the mutant receptor tkv\textsuperscript{6} was near the threshold for proper patterning of the imaginal discs. It was therefore an appropriate genetic background for identifying new components essential for mediating dpp signaling.

Enhancers of tkv are phenotypically similar to dpp mutants

The modifier screen was conducted as outlined in Fig. 2. The F\textsubscript{2} progeny were screened for enhanced phenotypes in the imaginal-disc-derived structures. Over 10,000 mutagenized genomes were screened and fourteen dominant enhancers defining seven loci were recovered (Table 1). The enhancers were recessive lethal in a wild-type background. tkv\textsuperscript{6} homozygotes that are heterozygous for the enhancer mutations had defects in imaginal disc development (Fig. 3). During pupal development, the dorsal proximal region of the two wing imaginal discs fuse to form the adult notum. In tkv\textsuperscript{6} flies, the notum appeared normal with a smooth contour and orderly oriented sensory bristles (Fig. 3A). This pattern was disrupted by heterozygous D1 mutation, resulting in a medial cleft in the notum, with abnormally parted bristles on both sides of the cleft (Fig. 3B). tkv\textsuperscript{6} flies had normally patterned legs (Fig. 3C). However, heterozygous D4 mutation caused deletions of distal and dorsal structures (Fig. 3D,E) and occasional duplication of ventrolateral structures such as sex combs on male prothoracic legs (Fig. 3E). These phenotypes were indistinguishable from

### Table 1. Summary of enhancer mutations

<table>
<thead>
<tr>
<th>Complementation group</th>
<th>Enhancement</th>
<th>Chromosomal location</th>
<th>Number of alleles</th>
<th>Allelic to</th>
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<tr>
<td>D1</td>
<td>notum</td>
<td>second</td>
<td>1</td>
<td>–</td>
</tr>
<tr>
<td>D2</td>
<td>notum</td>
<td>second</td>
<td>1</td>
<td>–</td>
</tr>
<tr>
<td>D3</td>
<td>legs and notum</td>
<td>second</td>
<td>5 (D3, D14, D15, D16, D24)</td>
<td>Mad</td>
</tr>
<tr>
<td>D17</td>
<td>legs and notum</td>
<td>second</td>
<td>1</td>
<td>tkv</td>
</tr>
<tr>
<td>D4</td>
<td>legs and notum</td>
<td>second</td>
<td>3 (D4, D8, D20)</td>
<td>66A</td>
</tr>
<tr>
<td>D5</td>
<td>legs and notum</td>
<td>third</td>
<td>1</td>
<td>Med</td>
</tr>
<tr>
<td>D13</td>
<td>legs and notum</td>
<td>third</td>
<td>2 (D13, D18)</td>
<td>punt</td>
</tr>
</tbody>
</table>

The assignment of the enhancer mutations to different complementation groups is based on genetic mapping and inter se complementation tests among enhancer mutants as described in Materials and Methods.
those of dpp<sup>disk</sup> alleles (Spencer et al., 1982), suggesting that these enhancers act in the dpp signal transduction pathway.

Additional evidence that the enhancers mediate dpp signaling came from their dosage-sensitive interactions with known mutations in the dpp pathway, including dpp<sup>35</sup>, tkv<sup>6</sup>, Df(2L)tkv<sup>2</sup>, sax<sup>5</sup>, punt<sup>145</sup>, Med<sup>60A</sup>, Med<sup>60D</sup> and shu<sup>16</sup> (Table 2). In many cases, the enhancers failed to fully complement these mutations and showed imaginal disc development defects ranging from gaps in wing veins to the notum and leg phenotypes described in Fig. 3. tkv<sup>6</sup> alone showed no detectable heterozygous interactions with the dpp pathway mutations examined except for the thickened venation phenotype when in trans to tkv<sup>6</sup> or Df(2R)tkv<sup>2</sup>, indicating that those phenotypes observed were due to the presence of the enhancer mutations.

**Dominant enhancers of tkv<sup>6</sup>: new alleles of tkv, Mad, Med, punt and 60A**

Meiotic mapping and complementation tests established seven complementation groups for the enhancers. As expected based on the initial evaluation of the tkv<sup>6</sup> genetic background, new alleles of tkv, Mad, Med and punt were recovered (Table 1). We sequenced the coding regions of the new Mad and punt alleles to establish their molecular identity. Of the five Mad alleles, three had point mutations in the coding region (Fig. 4A). Mis-sense mutations were found in both new punt alleles (Fig. 4B). The tkv<sup>D17</sup> and Med<sup>D5</sup> alleleism was based on genetic non-complementation. In addition, it was found that a tkv transgene rescued the lethality of tkv<sup>D17</sup> homozygotes, supporting the view that D17 is a tkv allele (data not shown).

Genetic and molecular characterizations of the D4 complementation group revealed that it corresponded to the 60A gene. 60A encodes a BMP-7 homologue isolated based on sequence homology (Doctor et al., 1992; Wharton et al., 1991). Its function remained unknown due to the lack of mutations in 60A. Three alleles of 60A were confirmed by sequencing the mutant alleles. 60A<sup>D8</sup> and 60A<sup>D20</sup> are nonsense mutations in the prodomain due to single nucleotide substitutions. 60A<sup>D14</sup> has one nucleotide deletion, causing a frame-shift premature stop also in the prodomain (Fig. 4C).

![Fig. 3. Heterozygous enhancer mutations enhanced tkv<sup>6</sup> phenotypes.](image)

(A) Phenotypically normal tkv<sup>6</sup> notum. (B) tkv<sup>D1</sup>/tkv<sup>6</sup> notum. The sensory bristles were parted to both sides and there was a profound medial cleft (arrow). The scutellum was often reduced in size (compare with A). (C) Phenotypically normal tkv<sup>6</sup> male prothoracic leg. The most distal tarsal segments and claws were truncated (arrow) and the ventral lateral sex combs were duplicated (arrow head). (E) tkv<sup>6</sup>/60A<sup>D14</sup> male thoracic leg. Note the severe truncation of distal structures and the curved appearance of the leg caused by the loss of dorsal tissues (arrow), fm, femur; th, tibia; sc, sex combs; ts, tarsal segments; cl, claws.
A. MAD

NH2  |  MH1  |  MH2  |  COOH

MadD14Q168*  |  MadD14Y255N  |  MadD20D437N

B. PUNT

punD13C67Y  |  punD10E378V

C. 60A

Leader  |  Prodomain  |  Mature domain

NH2  |  a  |  b  |  c  |  COOH

60A D60 Q137*  |  60A D60 W179*  |  60A D60 V237* (frame shift nonsense mutation)

Fig. 4. Molecular lesions in new alleles of Mad, punt and 60A. The position and nature of the mutations are indicated below the schematic diagram of the protein. Asterisk, nonsense mutation. (A) Mutations in new Mad alleles. The hatched box represents the mutation hot spot. (B) Mutations in new punt alleles. The structural features shown are the putative signal peptide (oval box), the extracellular cysteine residues (vertical bars), the transmembrane domain (hatched box) and the kinase domain (open box). (C) Mutations in 60A alleles. Letter Cs within the mature domain represent conserved cysteine residues.

Loss-of-function phenotypes of 60A

Animals lacking 60A function died at late larval/early pupal stages. One of the striking phenotypes of 60A mutant larvae is a transparent appearance due to the lack of fat body (Fig. 5B). In roughly 50% of the 60A larvae, the gastric caecae were reduced in length (Fig. 5D), consistent with the expression of 60A in the gastric caecae (Doctor et al., 1992). These phenotypes are similar to those of the Mad mutant larvae (Sekelsky et al., 1995).

During embryogenesis, 60A is expressed throughout the visceral mesoderm of the developing midgut (Doctor et al., 1992) suggesting a function for 60A in gut development. Indeed, embryos lacking 60A failed to form the first constriction (Fig. 6E,F). The homeotic gene Antennapedia (Antp) is expressed in the visceral mesoderm around the first constriction and is required for its formation (Reuter and Scott, 1990; Tremml and Bienz, 1989). We examined the Antp expression in 60A mutant embryos. Consistent with the lack of the first constriction, Antp expression was greatly reduced (Fig. 6B). Thus, 60A is required for the formation of the first constriction of the midgut, likely through positively regulating the expression of Antp in the visceral mesoderm.

60A maintains an optimal level of dpp signaling in the visceral mesoderm

The identification of mutations in 60A as dominant enhancers of tkv, thus dpp signaling, in the imaginal discs raised the possibility that 60A is required for optimal signaling by the dpp pathway. To determine if there was a general requirement for 60A in dpp signaling, we examined the effects of 60A mutations on dpp signaling in the visceral mesoderm where both dpp and 60A are expressed.

dpp is expressed in two discrete domains in the visceral mesoderm (Panganiban et al., 1990b). The anterior domain of dpp coincides with the gastric caecae primordia, which are immediately anterior to the expression domain of Sex combs reduced (Scr) in parasegment (ps) 4. The failure to initiate dpp expression in ps3 in dpp6 mutants results in anterior expansion of Scr expression and arrested outgrowth of the gastric caecae (Panganiban et al., 1990b; Hursh et al., 1993), indicating a role for dpp in repressing Scr expression during midgut development. During embryogenesis, dpp maintains an optimal level of Scr expression and arrested outgrowth of the gastric caecae (Panganiban et al., 1993).

During embryogenesis, dpp maintains an optimal level of Scr expression and arrested outgrowth of the gastric caecae (Panganiban et al., 1993).

Fig. 5. Larval phenotypes of 60A. (A) Wild-type third instar larva. (C) 60A D60/60A D60 third instar larva had greatly reduced fat body and appeared transparent. (B) Wild-type third instar larval gut. Note the long and extended gastric caecae (arrowhead). (D) 60A D60/60A D60 third instar larval gut. The gastric caecae were short in about half of the 60A larvae (n=47) (arrowhead). Compared with the wild-type larva of the same stage, 60A mutants grew more slowly and were somewhat reduced in size.
endoderm and formed three constrictions indicated by numbers. Antp and tkv expression of in ps6 in homozygous embryos (bracket).

Fig. 6. 60A mutants lacked the first constriction of the embryonic midgut. Lateral views of embryos stained with anti-Antp antibody (A-C) and anti-Lab antibody (D-F); stage 14 (A, B), stage 15 (C), stage 16 (D-F). Anterior is to the left and dorsal is up for this and subsequent figures. (A) Phenotypically normal visceral mesoderm expression of Antp in ps6 in tkv homozygous embryos (bracket). (B) 60A/60A embryos lacked ps6 Antp expression (bracket). (C) tkv/60A embryos lacked Antp expression in ps6 (bracket) but acquired an ectopic Antp domain in ps7 (arrowheads) presumably due to lacking Ubx expression in ps7 (see Fig. 7L). (D) tkv homozygotes had normal lab expression (arrowhead) in the endoderm and formed three constrictions indicated by numbers. (E) 60A/60A embryos failed to form the first constriction (asterisk) but the second and third constrictions still formed. lab expression was not altered (arrowhead). (F) tkv/60A/60A embryos lacked lab expression (arrowhead) in the endoderm and only formed one constriction.

Fig. 7. 60A enhanced tkv midgut phenotypes. Lateral views of embryos stained with anti-Scr antibody (A, E, I); anti-Dpp antibody (B, F, J); anti-Wg antibody (C, G, K) and anti-Ubx antibody (D, H, L). A-D, tkv homoyzogotes; E-H, 60A/60A embryos; I-L, tkv/60A/60A embryos. C, G and K were at stage 14; all other embryos were at stage 15. Scr expression in ps4 was normal in tkv/60A embryos; Scr expression in tkv/60A embryos, Scr extended anteriorly into ps3 (I, bracket). The visceral mesoderm expression of dpp in the single mutants were normal (B, F, arrows, gastric caeca; arrowheads, ps7). Double mutants had greatly reduced expression of dpp in gastric caeca (J, arrow) and in ps7 (J, arrowhead). dpp expression in the other domains were unaffected. Wg was expressed normally in the single mutants (C, G, arrowhead), however, ps8 expression is undetectable in the double mutants (K, arrowhead). Ubx expression in ps7 was normal in tkv/60A (D, bracket) or 60A mutants (H, bracket), but was greatly reduced in the double mutants (L, bracket).
reduction of dpp in ps3 in the double mutants may explain the observed derepression of Scr.

Ubx is required for repressing Antp in ps6. In Ubx mutants, the Antp domain is extended posteriorly into ps7 (Tremml and Bienz, 1989), indicating a homeotic transformation of ps7 into ps6. A similar phenotype was observed for tkv null embryos (Affolter et al., 1994). Consistent with the argument that lacking 60A compromises dpp signaling, there was also a posterior expansion of Antp in tkv60A double mutants (Fig. 6C). Interestingly, due to the 60A mutation, the endogenous Antp expression was absent, such that there was only ectopic Antp in ps7, where Ubx would normally be.

We also examined the expression of lab in the endoderm. Consistent with the gene expression changes in the visceral mesoderm, lab expression was not affected by tkv6 or 60A mutations. However, it was greatly reduced in tkv60A double mutants (Fig. 6F). The gene expression of the double mutants only formed two chambers instead of the normal four chambers (Fig. 6G, compare to 6D). This phenotype likely resulted from the failure to form the first constriction due to lacking 60A function and the failure to form the second constriction due to lacking dpp signaling. It is unclear why the position of the only constriction observed in the double mutants is somewhat more anterior than a normal third constriction.

The gene expression changes in the midgut of the tkv60A double mutants are consistent with 60A playing a role in augmenting dpp signaling.

**60A enhances the ectodermal phenotypes of tkv6 homozygotes**

Previous studies have established dpp's role as a morphogen in patterning the embryonic ectoderm (Ferguson and Anderson, 1992a,b; Wharton et al., 1993). dpp signaling is also required for dorsal closure of the embryonic ectoderm (Hou et al., 1997; Riesgo-Escovar and Hafen, 1997). We therefore examined if the level of 60A affected the phenotype of the embryonic ectoderm.

We compared the cuticle phenotypes of single and double mutants. Since tkv6 homozygotes were viable and 60A mutants had no obvious defects until late in development, the cuticular patterns of these mutants were essentially normal (Fig. 8A, B). However, tkv60A homozygote embryos died and exhibited head defects and an excessive ventral curvature (Fig. 8C). Although the double mutant cuticles bore some resemblance to hypomorphic dpp mutants, they did not exhibit an obvious expansion of the ventral denticle belts (Wharton et al., 1993). The double mutant phenotype suggested, however, that, when dpp signaling was compromised in the embryonic ectoderm, removing 60A activity further attenuated dpp signaling. We considered that the relatively mild phenotype of the double mutant embryo might reflect partial rescue by maternally contributed wild-type Tkv receptors. Indeed, a quarter of the embryos produced by mothers homozygous for tkv6 and heterozygous for 60A exhibited a dorsal open phenotype similar (Fig. 8D) to that of zygotic tkv null embryos (Penton et al., 1994). Therefore, in the absence of maternally provided wild-type Tkv, tkv60A double mutant embryos exhibit a phenotype indicative of defective dpp signaling during the process of dorsal closure.

**DISCUSSION**

The haploinsufficiency of the dpp locus reflects the sensitivity of developmental processes to a reduction in dpp signaling. We have carried out a genetic screen to search for modifiers of tkv6, a hypomorphic type I dpp receptor. tkv has been implicated in all aspects of dpp signaling both in vitro and in vivo (Penton et al., 1994; Nellen et al., 1994; Brummel et al., 1994; Affolter et al., 1994; Burke and Basler, 1996; Singer et al., 1997). Therefore, the modifiers of tkv6 are most likely to be integral components of the dpp signal transduction pathway.

**Identification of new alleles of tkv, Mad, Med, punt, 60A and two new loci as dominant enhancers of tkv6**

New alleles of several genes known to mediate dpp signaling were identified, including tkv, punt, Mad and Med. Recovery of these mutations as dominant enhancers of tkv6 validates the specificity of the screen.

We isolated five alleles of Mad, a key signal transducer in dpp signaling (Newfeld et al., 1996; Kim et al., 1997). Three of them have point mutations in the coding region (Fig. 4A). The molecular lesions correlate with their phenotypic properties. MadD14 has a nonsense mutation predicted to produce a truncated protein with only the conserved MH1 domain. It behaves genetically as a null. MadD15 has a tyrosine-to-asparagine change in the divergent linker region and behaves as a hypomorph with residual activity (Table 2). This suggests that the amino acid change only partially affects the protein function. The MH2 domains of Smads are highly conserved.
The three-dimensional structure of the MH2 domain of Smad4 indicates that Smads form homotypic homo-oligomers, whose intact conformation is essential for the assembly of a heteromer with a different Smad homotrimer in response to receptor activation (Hata et al., 1994; Shi et al., 1997). Many of the Smad mutations associated with tumors or affecting development map to the MH2 domain. Based on the crystal structure of the carboxy terminal domain of Smad4 (Shi et al., 1997), the invariant aspartic acid acid mutated to asparagine in MadD24 maps to the trimer interface region critical for trimerization. The corresponding residue in Smad2 is mutated in colon cancers (Eppert et al., 1996). Interestingly, the MadD24 mutation resulted in dominant female sterility (Y. C. and F. M. H., unpublished data) which is not observed with Mad null alleles, suggesting that it has a dominant negative effect, perhaps by forming inactive oligomers with the wild-type proteins in the heterozygotes.

Analysis of the two new punt alleles also provides evidence for the in vivo importance of conserved structural motifs in this type II dpp receptor (Fig. 4B). puntD13 has a cysteine-to-proline change in the extracellular cysteine cluster characteristic of all receptors for TGF-β superfamily members (Massagué et al., 1994), puntD18 changes the highly conserved glutamic acid to a valine in the catalytic core of the kinase domain, where another punt mutation, punt135, is mapped (Ruberte et al., 1995). Like punt135, both new punt alleles display some temperature sensitivity (Y. C. and F. M. H., unpublished data), suggesting that they are not protein nulls. No null mutations in the punt locus have been reported, suggesting that like dpp, punt may be haploinsufficient.

We did not isolate any new alleles of shn, which enhanced tkv in the initial test. The enhancer by shn heteroalleles may be allele specific, such that a particular form of mutant Shn protein is needed to produce an enhancement. Consistent with this, shn+, which makes no detectable protein (Staehling-Hampton et al., 1993), failed to enhance the tkv phenotype (data not shown). No dpp alleles were recovered either, possibly due to the haploinsufficiency associated with the locus and the fact that most hypomorphic dpp mutations affect regulatory regions, which are less likely to be affected by chemical mutagens such as ENU.

One unexpected locus identified in our screen is 60A, which encodes the BMP-7 homologue (Doctor et al., 1992; Wharton et al., 1991). The fact that in a screen of the entire genome, 60A mutations were recovered multiple times as dominant enhancers of a mutant dpp receptor provides strong evidence for its involvement in dpp signaling. Nonsense mutations were found in all three alleles of 60A in the prodomain of the precursor protein. Since these mutations are predicted to eliminate translation of the biologically active mature C-terminal domain, they most likely represent functional nulls of the 60A gene.

The developmental functions of 60A and its role in dpp signaling

Phenotypic analysis of 60A single mutants and tkv 60A double mutants revealed both dpp-independent and dpp-dependent functions for 60A. 60A is expressed broadly throughout development, with enrichment in the developing gut (Doctor et al., 1992), suggesting a role for 60A in gut morphogenesis. 60A mutants lack the first constriction of the embryonic midgut and Antp expression in ps6, indicating that 60A is required for the formation of the first constriction, possibly through regulating Antp expression. This function is independent of dpp signaling, since mutations in dpp or its receptors only disrupt the formation of the second but not the first constriction (Panganiban et al., 1999; Nellen et al., 1994; Ruberte et al., 1995). This also suggests that there is either redundancy or that a different receptor system is responsible for mediating 60A signaling to pattern the first constriction. It would be interesting to see if Atr (Childs et al., 1993), a type I receptor and STK- D (Ruberte et al., 1995), a type II receptor in Drosophila, both of unknown function, are mediators of 60A signaling at the site of the first constriction.

The fact that 60A mutations are dominant enhancers of a sensitized dpp pathway implicates 60A in potentiating dpp signaling. This is most obvious in the visceral mesoderm of the midgut where dpp signaling is required to regulate homeotic gene expression and to maintain its own expression through a positive feedback mechanism. Although dpp signaling in the visceral mesoderm appears intact in 60A mutants, a requirement for 60A is revealed in tkv 60A double mutants. When dpp signaling is attenuated by a mutant tkv receptor, eliminating 60A function reduces the signaling to below threshold level. The derepression of Scr in the anterior midgut and the loss of expression of dpp target genes, wg, Ubx and dpp, in the visceral mesoderm and lab in the endoderm are consistent with inadequate dpp signaling. A similar requirement for 60A is observed during dorsal closure of the embryonic ectoderm. The enhanced phenotypes of the adult appendages closely resemble those of the dpp hypomorphic mutants (Spencer et al., 1982), suggesting that 60A activity is also required for imaginal disc patterning. It is interesting that the imaginal discs are more sensitive to the reduction of 60A function, as a 50% reduction in 60A function is sufficient to produce a phenotype in a tkv 60A genetic background. This may reflect a differential threshold requirement for dpp signaling in different tissues. Taken together, our data argue for an involvement of 60A in dpp signaling at different developmental stages and in various tissues.

In a signaling system with multiple interacting dimeric ligands, the interpretation of any single mutant phenotypes must consider the effect of losing both homomeric and possible heteromeric ligands. Therefore, the functions of the dpp pathway may be a composite input from Dpp homodimers, and Dpp/Swc and Dpp/60A heterodimers. Alternatively, 60A homodimers may function in an additive fashion with Dpp homodimers at sites of overlapping expression. However, the loss-of-function phenotypes of dpp are as severe as the loss-of-function phenotypes of its downstream components, such as tkv or Mad (Padgett et al., 1987; Penton et al., 1994; Nellen et al., 1994; Newfeld et al., 1996, 1997), suggesting that there is very little signaling, if any at all, from 60A homodimers in dpp-dependent events. Therefore, we believe it is unlikely that 60A homodimers play a significant role in dpp-dependent processes. Rather, we favor the interpretation that Dpp/60A heterodimers form at sites of overlapping expression and participate with Dpp homodimers in multiple signaling events.

The overlapping expression patterns of murine BMPs have led to the suggestion that they may act combinatorially during development (Lyons et al., 1995). Given the dimeric nature of TGF-β superfamily ligands, one mechanism to achieve such a
combinatorial effect is to form functional heterodimers. Heterodimers of Xenopus BMP-4 and BMP-7 have been generated in vitro (Hazama et al., 1995) and shown to be more potent in bone- (Aono et al., 1995) and mesoderm-inducing assays (Suzuki et al., 1997) than either homodimer. In Drosophila, the Scw protein is proposed to upregulate dpp activity by forming Dpp/Scw heterodimers in the dorsal/ventral patterning of the embryonic ectoderm (Arora et al., 1994). The broad distribution of 60A proteins provides an opportunity for forming Dpp/60A heterodimers. Unlike scw null mutations, no obvious disruption of dpp signaling is observed in 60A null mutants, suggesting that Dpp/60A heterodimers are not as limiting as Dpp/Scw heterodimers, but partially redundant with Dpp homodimers. The first constriction phenotype of 60A mutants is unique, suggesting that it may be a function of 60A homodimers.

The tkv$^6$ receptor failed to bind homomeric ligands when expressed in COS cells (Penton et al., 1994). Since dpp signaling is intact in tkv$^6$ mutants, it would be interesting to determine if the mutant receptor still binds Dpp/60A and/or Dpp/scw heterodimers. If this is the case, the severe midgut phenotypes of tkv$^6$/60A double mutants could be due to additional loss of signaling from Dpp/60A heterodimers, which would be consistent with the proposal that 60A potentiates dpp signaling by forming heterodimers with Dpp. The cuticular phenotypes of tkv$^6$/60A double mutants without maternal wild-type Tkv receptor do not exhibit the altered dorsal/ventral polarity observed in dpp null embryos, possibly due to Dpp/Scw heterodimer signaling through the tkv$^6$ receptor during early embryogenesis.

In summary, we have isolated mutant alleles of genes involved in dpp signaling, including 60A. Mutations in 60A disrupt dpp signaling in multiple developmental processes when dpp signaling is compromised. We propose that 60A participates in dpp signaling by forming heterodimers with Dpp protein. Our data support both dpp-dependent and dpp-independent functions for 60A. It remains to be determined if these distinct functions reflect the qualitative differences between different forms of the ligands and if they are mediated by differentially activated receptors or distinct cytoplasmic signal transducers. The availability of 60A mutations provides a genetic tool for dissecting the differential requirement of each component in this combinatorial signaling system.

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