

***Mothers against dpp* encodes a conserved cytoplasmic protein required in DPP/TGF- β responsive cells**

Stuart J. Newfeld¹, Elena H. Chartoff^{1,†}, Jonathan M. Graff², Douglas A. Melton² and William M. Gelbart^{1,*}

¹Department of Molecular and Cellular Biology, ²Howard Hughes Medical Institute, Harvard University, Cambridge, Massachusetts 02138, USA

*Author for correspondence at The Biological Laboratories, Harvard University, 16 Divinity Ave., Cambridge, Massachusetts 02138, USA (e-mail: gelbart@morgan.harvard.edu)

[†]Present address: Behavioral Neuroscience Interdisciplinary Group, University of Washington, Seattle, Washington 98195, USA

SUMMARY

The proteins necessary for signal transduction in cells responding to ligands of the TGF- β family are largely unknown. We have previously identified *Mad* (*Mothers against dpp*), a gene that interacts with the TGF- β family member encoded by *decapentaplegic* (*dpp*) in *Drosophila*. Assay of *Mad*'s role in the DPP-dependent events of embryonic midgut development demonstrates that *Mad* is required for any response of the visceral mesoderm or endoderm to DPP signals from the visceral mesoderm. Replacement of the normal DPP promoter with a heterologous (*hsp70*) promoter fails to restore DPP-dependent responses in *Mad* mutant midguts. Experiments utilizing

Mad transgenes regulated by tissue-specific promoters show that MAD is required specifically in cells responding to DPP. Immunohistochemical studies localize MAD to the cytoplasm in all tissues examined. Experiments in *Xenopus* embryos demonstrate that *Drosophila* MAD can function in the signaling pathway of BMP-4, a vertebrate homolog of *dpp*. Based on these results, we propose that *Mad* is a highly conserved and essential element of the DPP signal transduction pathway.

Key words: *Mad*, intercellular signaling, TGF- β family, midgut morphogenesis, *Drosophila*

INTRODUCTION

Ligands of the transforming growth factor- β (TGF- β) superfamily are required for numerous developmental events in many organisms (reviewed in Kingsley, 1994). These secreted molecules function through the activation of receptor kinases on the surface of responsive cells (reviewed in Massagué et al., 1994). The activated receptors in turn are thought to propagate the signal through the initiation of intracellular signal transduction cascades. No protein components of these cascades have yet been firmly identified. Here, we report a strong candidate for such a component.

This candidate, the *Mothers against dpp* (*Mad*) gene in *Drosophila melanogaster*, was initially identified by virtue of dominant phenotypic interactions between *Mad* mutant alleles and specific alleles of the *decapentaplegic* (*dpp*) gene (Raftery et al., 1995; Sekelsky et al., 1995). The protein product of the *dpp* gene, which we refer to as DPP, is a member of the TGF- β family (Padgett et al., 1987) and appears to be the *Drosophila* homolog of the vertebrate BMP-2 and BMP-4 ligands (Padgett et al., 1993; Sampath et al., 1993). Among its many functions, DPP signaling across germ layers is responsible for the establishment of dorsal mesoderm (Staehling-Hampton et al., 1994; Frasch, 1995). Studies in *Xenopus* show that BMP-4 induces ventral mesoderm (reviewed in Harland, 1994), an activity suggested to be analogous by virtue of the hypothesized reversal of the dorsal-ventral axis after the divergence of

arthropods and vertebrates (Holley et al., 1995; Schmidt et al., 1995).

Significant progress has been made in characterizing participants in the DPP signaling pathway. Recent reports have identified the DPP type I and II receptors (Brummel et al., 1994; Nellen et al., 1994; Penton et al., 1994; Xie et al., 1994; Letsou et al., 1995; Ruberte et al., 1995), a potential regulator of DPP processing (*tolloid*, Shimmel et al., 1991; Finelli et al., 1994), and a transcription factor functioning in DPP-responsive cells (*schnurri*, Arora et al., 1995; Grieder et al., 1995; Staehling-Hampton et al., 1995). However, the cytoplasmic proteins necessary for transducing the DPP signal from cell surface receptors to nuclear transcription factors are largely unknown. To identify these proteins, our laboratory has conducted several genetic screens (Raftery et al., 1995; Sekelsky et al., 1995).

Mad and *dpp* display dosage-dependent genetic interactions and homozygous *Mad* mutant phenotypes show striking parallels with *dpp* mutant phenotypes. However, the predicted MAD polypeptide contains no identifiable protein motifs, providing no clues to its biochemical function (Sekelsky et al., 1995). Recently, three *C. elegans* genes (*sma-2*, *sma-3* and *sma-4*) were shown to have strong sequence similarity to *Mad* (Sekelsky et al., 1995; Savage et al., 1996). These genes also share aspects of the mutant phenotype displayed by the *C. elegans* TGF- β type II receptor *daf-4*. Clonal analysis of cells contributing to the shared mutant phenotype revealed that *sma-*

2 is required in the same cells as *daf-4*, yet no direct interaction between *sma-2* and *daf-4* was detected (Savage et al., 1996).

Numerous *Mad*-like sequences have been cloned from mammals (Savage et al., 1996) and *Xenopus* (Graff et al., 1996). Recently, a candidate tumor suppressor locus associated with human pancreatic cancers (*DPC-4*) was identified which shows extensive sequence similarity to *Mad* (Hahn et al., 1996). Thus, analyses of *Mad* may impact on our understanding of TGF- β -mediated events in many organisms.

MATERIALS AND METHODS

Drosophila strains

Strains carrying the mutations *lab^{vd1}*, *Ubx¹* and *dpp^{s4}* are described in Lindsley and Zimm (1992). The deficiency *Df(2L) C28*, which removes *Mad*, is described in Raftery et al. (1995). The sequenced point mutant null alleles *Mad¹⁰* and *Mad¹²* and the deficiency *Df(2L) JS17*, which removes *Mad*, are described in Sekelsky et al. (1995). Strains carrying the β -galactosidase reporter genes are described as follows: *lab* anterior midgut endoderm/CNS reporter P{3.65*lab66a*} (Chouinard and Kaufman, 1991), *dpp* visceral mesoderm reporter P{RD2} and *Ubx* visceral mesoderm reporter P{15-1} (Hursh et al., 1993), *Scr* anterior/posterior visceral mesoderm reporter P{HZR+0.8X/H} (Gindhart, Jr. et al., 1995), and a *wingless* enhancer trap P{*enlacZ*} on *CyO* (Kassis et al., 1992). Additional transgenic lines are described as follows: P{GawB}24B - a Gal4 enhancer trap line expressing in the mesoderm (Brand and Perrimon, 1993), P{*hs-dpp*.BP} - a line with three insertions on III (Twombly et al., 1996), and P{Um*Mad*}1ab - a line with two copies of the *myc*-epitope tagged *Mad* cDNA rescue construct on III (Sekelsky et al., 1995).

Embryo analysis strategies

Mad mutant experiments

For all reporter gene studies in *Mad* mutant backgrounds, both point mutants, *Mad¹⁰* and *Mad¹²*, and deficiencies removing *Mad* [*Df(2L) JS17* and *Df(2L) C28*], were balanced over a *CyO* chromosome containing an enhancer trap in *wingless* to enable positive identification of *Mad* mutant embryos. Every study was accompanied by an analysis of the reporter gene's parental strain to generate the wild-type expression pattern for comparison. For *lab* in *Mad* mutant backgrounds, with/without P{*hs-dpp*.BP}, stocks were constructed using the deficiencies *Df(2L) JS17* and *Df(2L) C28* which were also homozygous for the *lab* reporter gene on X and multiple P{*hs-dpp*.BP} insertions on III. These females were crossed to *Mad¹²* males and the transheterozygous *Mad* mutant embryos evaluated for *lab* expression. To induce P{*hs-dpp*.BP} expression in these embryos, 0-15 hour egg lays were heated to 37°C for 1 hour and then returned to 25°C for 4 hours before histochemical analysis. At least two independent heat-shock trials were conducted for both transheterozygous *Mad* mutant combinations. For *dpp* and *Ubx* visceral mesoderm reporter genes P{RD2} and P{15-1}, stocks were constructed using *Mad¹²* and homozygous viable insertions on III. For *Scr* visceral mesoderm reporter gene P{HZR+ 0.8X/H}, a stock was constructed using *Mad¹²* and an insertion on TM6B maintained over *Sb gl³*. Double labeling of embryos from a *Mad¹²* stock by RNA in situ hybridization with probes derived from *dpp* cDNA H1 and the β -galactosidase-expressing vector pSV- β -galactosidase (Promega) were performed as described (Ray et al., 1991).

Midgut endoderm enhancer characterization

For the analysis of P{*mex1* β gal} wild-type expression, three independent insertions were made homozygous. For P{*mex1* β gal} expression in *dpp* and *Mad* mutant backgrounds, mutant stocks

homozygous for an insertion on III were created. For P{*mex1* β gal} expression in *lab* and *Ubx* mutant backgrounds, mutant stocks homozygous for an insertion on the X were created.

MAD tissue-specific expression experiments

For *lab* in *Mad* mutant backgrounds, with/without P{*hs-dpp*.BP} and with MAD in the midgut endoderm, both lines of *Mad* deficiency-bearing females homozygous for P{3.65*lab66a*} on the X were crossed to *Mad¹²* males who were homozygous for a P{*mex1**Mad*} insertion on III. 100% of the progeny will contain the *lab* reporter P{3.65*lab66a*}, P{*hs-dpp*.BP} and P{*mex1**Mad*}. Three independent insertions of P{*mex1**Mad*} were tested. The heat-shock routine is as above. For *lab* in *Mad* mutant backgrounds, with/without P{*hs-dpp*.BP} and MAD in the visceral mesoderm, both lines of *Mad* deficiency-bearing males containing P{3.65*lab66a*} on the X were crossed to *Mad¹²* females who were homozygous for P{UAS*Mad*.N} on the X and homozygous for P{GawB}24B on III. 50% of the *Mad* mutant embryos will have the *lab* reporter. For embryos expressing MAD in the visceral mesoderm and midgut endoderm, males containing the *lab* reporter P{3.65*lab66a*}, P{*hs-dpp*.BP} and P{*mex1**Mad*} from the midgut endoderm experiment above were crossed to females homozygous for P{UAS*Mad*.N} on the X and homozygous for P{GawB}24B on III. 25% of the *Mad* mutant embryos will have the *lab* reporter and P{*mex1**Mad*}. For embryos expressing *Mad* in the visceral mesoderm and midgut endoderm with P{*hs-dpp*.BP}, the direction of this cross was reversed allowing the formation of recombinant chromosomes containing P{*hs-dpp*.BP} and P{*mex1**Mad*}. For P{RD2} in *Mad* mutant backgrounds with MAD in the visceral mesoderm, males from *Mad* point mutant stocks homozygous for P{RD2} were crossed to females containing the same *Mad* allele that were also homozygous for an insertion of P{UAS*Mad*.N} on the X and homozygous for P{GawB}24B on III. 100% of the progeny will contain one copy of P{UAS*Mad*.N}, P{GawB}24B and P{RD2}. Two insertions of P{UAS*Mad*.N} were tested.

P-element constructs

P{*mex1* β gal} was constructed from a 2.15 kb *EcoRI*-*DraIII* fragment from genomic clone *EcoRI**mex1*IG2 (R. Schulz, M.D. Anderson Cancer Center) which corresponds to map position +6 to +8 on the chromosome walk around *Eip28/29* (Cherbas et al., 1986). This fragment was cloned into the *EcoRI* and *BamHI* sites of pCasper-AUG- β gal (Thummel et al., 1988) after the *DraIII* and *BamHI* sites were filled in with T4 DNA polymerase. This fragment contains approximately 2 kb of upstream sequence, the promoter and all 5' untranslated sequence (*DraIII* cuts the ATG encoding the initiator methionine) for *midgut expression 1* (*mex1*; Schulz et al., 1991). P{UAS*Mad*.N} was constructed from the complete *Mad* cDNA (Sekelsky et al., 1995). The cDNA was first cloned into pSport1 (BRL) and then removed using *KpnI* and *XbaI* sites from the pSport1 polylinker. The cDNA was then cloned into pUAST (Brand and Perrimon, 1993) using these sites. P{*mex1**Mad*} was constructed from the same *KpnI*-*XbaI* fragment containing the *Mad* cDNA. In this case, the fragment was cloned into P{*mex1* β gal} which had been cut with *KpnI* and *PstI*, after the *XbaI* and *PstI* sites were filled in with T4 DNA polymerase. This replaces β -galactosidase sequences with the *Mad* cDNA. Three independent lines were established for each construct.

Histochemical β -galactosidase analysis

Embryos were collected and dechorionated by standard methods and fixed in 4% formaldehyde in PEM (0.1 M Pipes; 1 mM EGTA; 2 mM MgSO₄):n-heptane at 1:1. Embryos were washed in PEM and analyzed for β -galactosidase activity according to Blackman et al. (1991).

MAD antiserum and western blots

An *XhoI*-*AccI* (XA) restriction fragment from the *Mad* cDNA (bp 704-1524; Sekelsky et al., 1995) was subcloned into Bluescript

(Stratagene). This fragment was cut out with *Xho*I and *Hind*III, cloned into the pGEX-KG expression vector (Guan and Dixon, 1991) and transformed into *E. coli* host strain DH5 α . The XA-GST fusion protein was induced and purified according to Frangioni and Neel (1993). The 31 \times 10³ *M_r* XA polypeptide was cleaved from the GST-glutathione sepharose 4B matrix (Pharmacia) according to manufacturer's instructions. The XA fragment was then isolated by SDS-PAGE (Laemmli, 1970). The 1% acrylamide gel was briefly stained in Coomassie Blue, the XA band excised and soaked in PBS (342 mM NaCl, 6.7 mM KCl, 3.7 mM KH₂PO₄, 10.4 mM NaH₂PO₄) at 4°C. The gel slice was used to immunize two female guinea pigs (890 and 891) according to standard protocols (Pocono Rabbit Farm).

Protein from adult female *y w* and P{Um*Mad*}*lab* flies was extracted by grinding 20 flies in 100 μ l PBS and 100 μ l 2 \times SDS gel loading buffer (Sambrook et al., 1989). The samples were boiled for 5 minutes and approximately 1 fly worth of protein was loaded per lane. The gel was soaked and electroblotted to nitrocellulose according to Thomas and Kiehart (1994). Membranes were blocked in TBS (150 mM NaCl, 10 mM Tris pH 7.5, 0.1% BSA, 0.1% sodium azide)/10% powdered milk for 2 hours at 25°C and then incubated with various dilutions of anti-MAD polyclonal serum or with mouse anti-MYC monoclonal antibodies at a 1:10 dilution in TBST (TBS with 0.1% Triton X-100)/10% normal goat serum (NGS, Sigma) for 1 hour at 25°C. Blots were washed in TBST 3 times for 10 minutes and then incubated with alkaline phosphatase (AP)-conjugated goat anti-guinea pig antibody diluted 1:5000 in TBST/10% NGS for 1 hour at 25°C or with AP-conjugated goat anti-mouse antibodies at a 1:1000 dilution in TBST/10% NGS for blots incubated with anti-MYC antibodies. After washing in TBST and rinsing in TBS, Western Blue Stabilized Substrate for AP (Promega) was used to visualize labeled protein. Proteins from dissected salivary glands of third instar P{Um*Mad*}*lab* larvae were extracted and blotted as above.

Antibody analyses of salivary glands and embryos

The primary antibodies are anti-MYC monoclonal antibodies (Oncogene Science 9E10) used on salivary glands at 1:20 and anti-MAD polyclonal serum (guinea pig 891) used on salivary glands and embryos at 1:2000. The secondary antibodies are fluorescein-conjugated goat anti-guinea pig (Capell Research Products) and fluorescein-conjugated goat anti-mouse (Jackson ImmunoResearch Laboratories) used at 1:200. Salivary glands were dissected from climbing third instar larvae in ice-cold PBS, fixed for 15 minutes in 4% formaldehyde in PEM and washed three times for 10 minutes in PBT (PBS with 0.5% Triton X-100, pH 7.0). Salivary glands were blocked in PBT/10% NGS at room temperature for 1 hour. Following a rinse in PBT/10% NGS, salivary glands were incubated with primary antibody (diluted in this solution) overnight at 4°C. All subsequent treatments followed Thomas and Kiehart (1994).

Embryos were collected, fixed as described for histochemical analysis, rehydrated from methanol into PBT and blocked in PBT/10% NGS/0.5% BSA (bovine serum albumin fraction V, Sigma). Following a rinse in PBT/5% NGS/0.25% BSA, the remaining steps are identical to those for salivary glands. Anti-MYC incubation of salivary glands from P{Um*Mad*}*lab* larvae was always accompanied by salivary glands from *y w* larvae as a negative control and 42 π M transgenic larvae (expressing *myc*-tagged P-element transposase; Xu and Rubin, 1993) as a positive control. Analyses with anti-MAD serum were always accompanied by preimmune serum as a negative control.

Xenopus methods

To generate injectable RNA that encoded *Drosophila* MAD, the *Nco*I-*Stu*I restriction fragment from the P{Um*Mad*} plasmid (Sekelsky et al., 1995) was subcloned into the *Not*I site of pSP64TEN. This construct, pSP64TEN-*myc*MAD, was linearized with *Xba*I and synthetic capped mRNA generated as described by Krieg and Melton (1987). mRNAs encoding *Xenopus* BMP-4, the BMP-4 type I receptor

or the activin type II receptor were synthesized as described (Graff et al., 1994; Hemmati-Brivanlou and Melton, 1992). Embryos were injected, staged and analyzed as described (Graff et al., 1994).

RESULTS

Examining the role of *Mad* in the DPP pathway

To clarify *Mad*'s function, we employed *dpp*'s role in the formation of the embryonic second midgut constriction (reviewed in Bienz, 1994) as our primary assay. In the visceral mesoderm of embryonic parasegment 7 (ps7), the homeotic gene *Ultrabithorax* (*Ubx*) initiates *dpp* expression. Then DPP signals between germ layers to the underlying midgut endoderm. At the same time, within the visceral mesoderm, DPP functions in an autocrine manner to maintain *Ubx* and thus *dpp* expression. Within the ps7 midgut endoderm, which does not express *dpp*, transcription of the homeotic gene *labial* (*lab*) is dependent upon the DPP signal. Each of these genes is required for the second constriction. *Mad* is also required for the second constriction and is transcribed in both cell layers (Sekelsky et al., 1995). A schematic of the known interactions between *dpp*, *Ubx* and *lab*, as well as possible roles for *Mad* in ps7 is shown in Fig. 1A.

Here we report the results of two classes of experiments. First, using epistasis tests, we determined where *Mad* functions with regard to *dpp* transcription. Second, using tissue-specific expression of MAD in otherwise *Mad* mutant embryos, we determined which cells require MAD for DPP-dependent responses.

Mad functions downstream of *dpp* transcription

Expression of a *lab* reporter gene containing a DPP-independent central nervous system (CNS) enhancer and a DPP-dependent ps7 midgut endoderm enhancer in a wild-type (*Mad*⁺) background is shown in Fig. 1B. Comparably staged *Mad* mutant embryos retain the *lab* CNS expression but lack *lab* expression in the midgut endoderm (Fig. 1C). This result is consistent with previous observations that *lab* RNA in the midgut endoderm was absent in *Mad* mutant embryos (Sekelsky et al., 1995). To test if the role of *Mad* is to regulate *dpp* transcription, we assayed the effect of *Mad* mutations on *lab* induction in embryos in which *dpp* transcription was placed under control of the *Drosophila* hsp70 promoter (P{hs-*dpp*.BP}; Twombly et al., 1996). Under these conditions, if *Mad* functions upstream of *dpp* transcription, *lab* expression in the midgut endoderm should occur in *Mad* mutant embryos. However, the expanded domain of *lab* midgut endoderm expression expected from using P{hs-*dpp*.BP} in *Mad*⁺ embryos (Fig. 1D; Thuringer and Bienz, 1993) is lacking in embryos that are mutant for *Mad* (Fig. 1E). Thus, we infer that *Mad* functions in the DPP pathway between the initiation of *dpp* transcription and *lab* expression.

This idea is supported by experiments using the *dpp* reporter gene P{RD2} which accurately reflects *dpp* expression in the visceral mesoderm of ps3 and ps7 (Hursh et al., 1993). In ps7, the maintenance of *dpp* expression is controlled by an autocrine signaling pathway requiring DPP and UBX (Fig. 1A). There is no difference in the initiation of *dpp* expression between wild-type and *Mad* mutant embryos in either ps3 or ps7 (Fig. 2A,B). In wild-type embryos, *dpp* expression

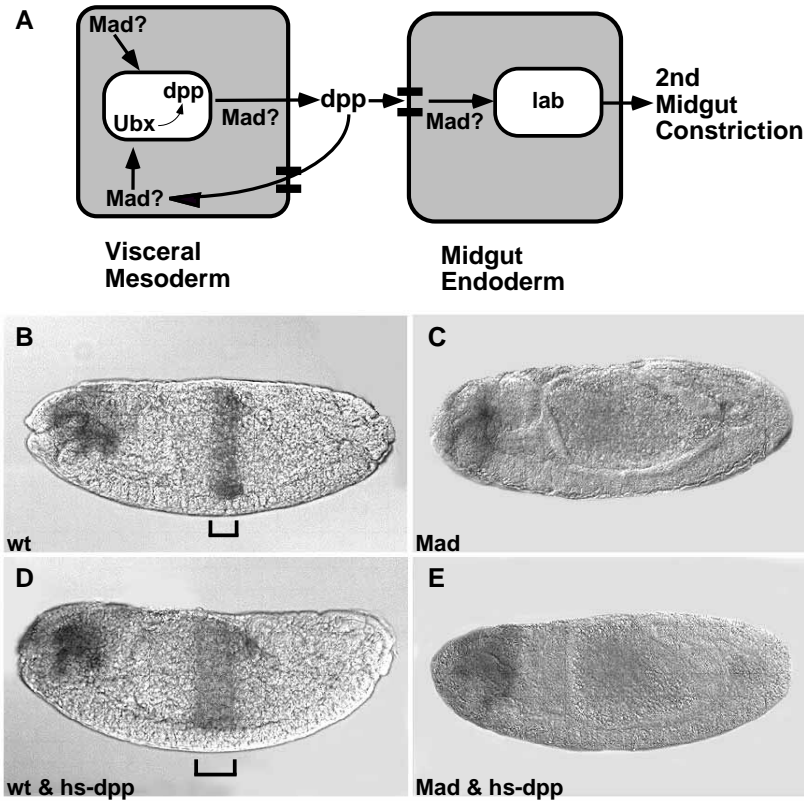


Fig. 1. MAD functions between *dpp* transcription initiation and *lab* transcription. (A) The known relationships between *Ubx*, *dpp* and *lab* in ps7 of the midgut and four possible functions for *Mad*. (B,D) Wild-type embryos; (C,E) *Mad* mutant embryos. (B,C) Embryos that were not heat shocked; (D,E) Embryos heat shocked to express ubiquitous DPP from P{*hs-dpp*.BP}. (B) Wild-type expression of a *lab* reporter gene which contains a DPP-independent central nervous system (CNS) enhancer and a DPP-dependent midgut endoderm enhancer. *lab* expression in the head and midgut endoderm (square bracket) are clearly seen. (C) CNS expression is unaffected in a *Mad* mutant embryo but no expression from the *lab* midgut endoderm enhancer is detectable. (D) Anterior expansion of *lab* midgut endoderm expression (extended square bracket) in response to P{*hs-dpp*.BP}. (E) P{*hs-dpp*.BP} expression has no effect on *lab* expression in *Mad* mutant embryos.

continues at very high levels in both parasegments through very late stages of gut elongation (Fig. 2C,E). In *Mad* mutant embryos, this is not observed; *dpp* expression begins to diminish shortly after initiation (Fig. 2D,F). We believe that perdurance of β -galactosidase is responsible for residual staining in these mutant embryos. RNA in situ hybridization studies using the *dpp* cDNA as a probe on *Mad* mutant embryos revealed only a short period of *dpp* transcription (data not shown). It appears that maintenance but not initiation of *dpp* expression requires *Mad*.

Analyses of *Ubx* and *Sex combs reduced* (*Scr*) visceral mesoderm reporter genes in *Mad* mutant embryos provides further evidence for placing *Mad* downstream of *dpp* transcription initiation. Initiation of *Ubx* expression in ps7 occurs in wild-type and *Mad* mutant embryos (Fig. 3A,B). Maintenance of *Ubx* expression in ps7 does not occur in *Mad* mutant embryos (data not shown). As in ps7, the maintenance of high levels of *dpp* expression in ps3 is not seen in *Mad* mutant embryos (compare Fig. 2C,E with D,F). Failure to initiate *dpp* expression in ps3 in *dpp* mutants which lack the visceral mesoderm expression of DPP results in an anterior expansion of *Scr* expression (Hursh et al., 1993). Similarly, *Mad* mutant embryos show an anterior expansion of *Scr*, using a reporter gene assay (Fig. 3C,D). It appears that MAD functions between initiation of *dpp* transcription and the repression of *Scr* in the visceral mesoderm of ps3. Thus, in all of its roles in embryonic midgut development, MAD functions downstream of *dpp* transcription initiation.

Midgut tissue-specific enhancers

Further resolution of MAD's role in the DPP pathway emerges from a determination of whether MAD is required in cells

sending or receiving a DPP signal. We have focused on the DPP-dependent induction of *lab* expression, since the signaling cells are in the visceral mesoderm and the target cells are in the adjacent endoderm. To do this, we used tissue-specific enhancers to drive *Mad* expression only in the visceral mesoderm or only in the midgut endoderm. We then examined the ability of visceral mesoderm-expressed or midgut endoderm-expressed *Mad* to rescue *lab* induction in genetic backgrounds lacking endogenous *Mad* activity.

At this time, only a limited set of identified enhancer elements or enhancer traps that specifically express in the visceral mesoderm or midgut endoderm are available. Thus, we had to exploit enhancer constructs in special contexts. The properties of the enhancers that we selected are described here. To express MAD specifically in the visceral mesoderm, we utilized a Gal4 enhancer trap (P{GawB}24B; Brand and Perrimon, 1993) to drive expression of a *Mad* transgene carrying upstream activation sequences regulated by Gal4 (P{UAS-*Mad*.N}). Early expression from this enhancer trap occurs throughout the presumptive mesoderm while later expression becomes restricted to the somatic mesoderm. Utilizing this enhancer to drive MAD expression in the presumptive mesoderm provides MAD protein, which persists in descendant visceral mesoderm cells.

For midgut endoderm expression, we utilized an enhancer element upstream of *midgut expression 1* (*mex1*; Schulz et al., 1991) which drives expression only in the midgut endoderm. Reporter gene expression from P{*mex1* β gal} in *Mad*⁺ embryos is shown in Fig. 4A,C,E. Expression begins in ps7 and the surrounding midgut endoderm prior to the initiation of the second midgut constriction (at roughly the same time as *lab*) and continues strongly into the first larval instar. At all times, *mex1*

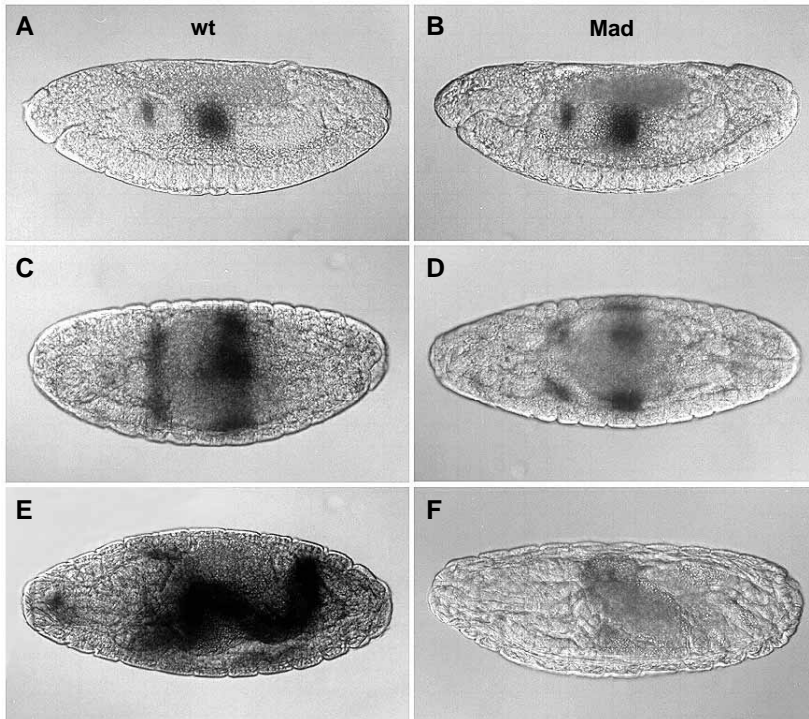


Fig. 2. MAD functions in *dpp* maintenance in the midgut. (A-F) Temporal studies of *dpp* reporter gene P{RD2}. The left panels show wild-type expression and the right panels expression in *Mad* mutant embryos. Comparison of A with B reveals that the initiation of *dpp* expression at stage 12, in ps3 and ps7, is unaffected in *Mad* mutant embryos. Comparison of the stage 15 embryo in C with D and the older embryo in E with F shows that *dpp* expression is maintained for an extended period at very high levels in both regions in wild-type but not in *Mad* mutants. All embryos were treated in parallel. It appears that *Mad* function is not required for *dpp* transcription initiation in ps3 and ps7 visceral mesoderm but for maintenance of *dpp* expression.

enhancer expression in the midgut endoderm is broader than the *lab* midgut endoderm enhancer (described in Chouinard and Kaufman, 1991). However, only the anterior and posterior ends of expression from the *mex1* enhancer are functional in *lab* (Fig. 4B), *dpp* (Fig. 4D) and *Mad* (Fig. 4F) mutant embryos as well as in *Ubx* mutants (data not shown). Thus, a construct (P{*mex1Mad*}) containing this enhancer driving MAD expression, in an otherwise *Mad* mutant background, can generate an embryo containing *Mad*⁺ midgut endoderm cells at the anterior and posterior ends of the *mex1* enhancer domain.

MAD is required in DPP-responsive cells

To determine which midgut cells require *Mad* function, we examined the expression of *lab* and *dpp* reporter genes in *Mad* mutant embryos with only tissue-specific expression of MAD. Expression of MAD in the visceral mesoderm has no effect on *lab* expression in the midgut endoderm, even in the presence of ubiquitously expressed DPP (Fig. 5A). In these embryos, DPP-independent *lab* expression in the CNS is evident but no midgut endoderm expression is visible, just as in homogeneous *Mad* mutant embryos (Fig. 1E). This result suggests that *Mad*'s role in *lab* induction does not involve secretion of DPP from the visceral mesoderm or any other process occurring only in the signaling cell.

In contrast, expressing MAD solely in the midgut endoderm in the presence of ubiquitous DPP rescues ectopic *lab* expression in the midgut endoderm as shown by the staining of cells in the elongating gut (Fig. 5C). No midgut endoderm *lab* expression is seen in homogeneous *Mad* mutant embryos, even with ubiquitous DPP, at any stage (e.g., Fig. 1E). This limited amount of *lab* expression is presumably occurring in the *lab*-independent anterior domain of *mex1* expression and requires the heat-shock *dpp* construct to provide a DPP signal in this domain. Comparing *lab* expression in embryos with

MAD expressed in the visceral mesoderm to embryos expressing MAD in the midgut endoderm (Fig. 5A,C) leads us to conclude that MAD is required in cells receiving a DPP signal.

Examination of *dpp* expression in embryos with MAD expressed specifically in the visceral mesoderm (Fig. 5B) reveals that the autocrine loop for *dpp* maintenance in both ps3 and ps7 is partially rescued. Note the well-defined staining of the elongating gastric caecae and midgut, particularly in comparison to wild-type and homozygous *Mad* mutant embryos (Fig. 2E,F). Our interpretation is that the provision of MAD in the early mesoderm allows sufficient MAD activity to persist into the derived visceral mesoderm to rescue early stages of *dpp* maintenance during gut elongation.

The expansion of UBX expression in response to heat-shock-induced DPP (Thuringer et al., 1993) permits a broad domain of autocrine signaling in the visceral mesoderm, including cells adjacent to the anterior region of *mex1* expression in the endoderm. Thus, *Mad* mutant embryos expressing MAD in both the visceral mesoderm and midgut endoderm with heat-shock-induced DPP expression exhibit stronger *lab* induction (compare Fig. 5C and D). Presumably this occurs because the visceral mesoderm expression of MAD allows more DPP to be generated and secreted through the restoration of the autocrine loop, reinforcing the effects of the heat-shock-induced DPP signal. All of our observations support a role for MAD in cells receiving a DPP signal (Fig. 5E).

MAD is a ubiquitously expressed cytoplasmic protein

A very intriguing possibility is that MAD is a component of the signal transduction pathway in these cells. Knowing the subcellular localization of MAD would allow us to evaluate this possibility. Therefore, the subcellular localization of MAD

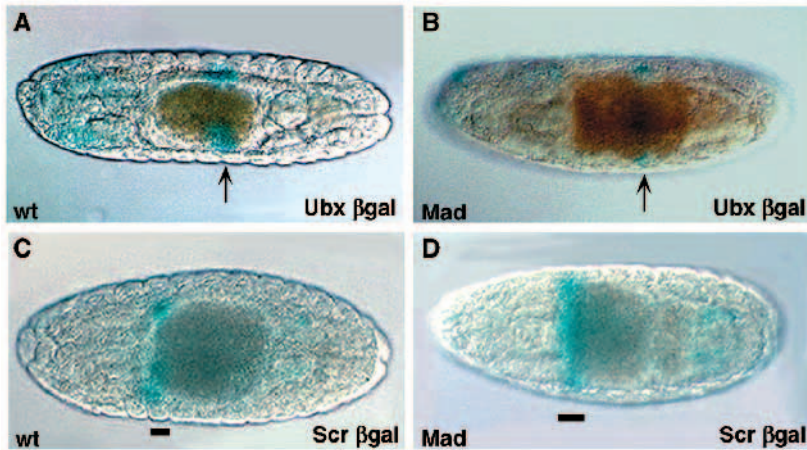


Fig. 3. *Ubx* and *Scr* expression in *Mad* mutant embryos. (A,B) Studies of a *Ubx* reporter gene. (C,D) Studies of an *Scr* reporter gene. The left panels show wild-type expression and the right panels expression in *Mad* mutant embryos. Comparison of A with B shows that the initiation of *Ubx* expression in ps7, indicated by an arrow, is observed in both embryos. Comparison of C with D shows that the normal ps4 expression of *Scr* in wild-type embryos (indicated by a horizontal bar) is expanded anteriorly to include ps3 in *Mad* mutant embryos (indicated by an extended bar).

protein was examined using immunohistochemical techniques. We obtained very similar results with two distinct antibody probes. One probe is anti-MYC monoclonal antibodies directed against a *myc*-epitope-tagged *Mad* cDNA transgene (Sekelsky et al., 1995). The other is anti-MAD polyclonal serum generated against a bacterially expressed fusion protein. One *myc*-epitope-tagged *Mad* transgenic line overexpresses the transgene in the larval salivary glands and their embryonic primordia (data not shown). Their accessibility and large size have made third instar larval salivary glands an excellent tissue for examining subcellular localization of proteins. This tissue also proved very useful for evaluating the specificity of our anti-MAD serum. The western strips in Fig. 6A were generated from extracts of salivary glands from transgenic larvae. Both anti-MYC monoclonal antibodies and anti-MAD polyclonal serum recognize a single protein of 55×10^3 Mr. The identified protein corresponds to the size of the predicted product

encoded by the *myc*-epitope-tagged *Mad* cDNA transgene carried in this strain. Preimmune serum does not recognize any proteins in these salivary glands. Fig. 6B,C are confocal micrographs of transgenic salivary glands incubated with anti-MYC monoclonal antibodies (Fig. 6B) and anti-MAD serum (Fig. 6C). In this tissue, MAD shows a cytoplasmic subcellular localization though some staining in a few nuclei is seen (Fig. 6C). However, we see no nuclear staining with anti-MYC antibodies or with anti-MAD on embryos (see below) suggesting that the nuclear staining does not accurately reflect MAD's subcellular localization. The transition in MAD staining, from uniform to punctate, in cells located further from the common duct reflects the accumulation of secretory granules in the cytoplasm (Berendes and Ashburner, 1978). Experiments with salivary glands from wild-type larvae reveal cytoplasmic staining with anti-MAD serum but no signal with anti-MYC monoclonal antibodies (data not shown).

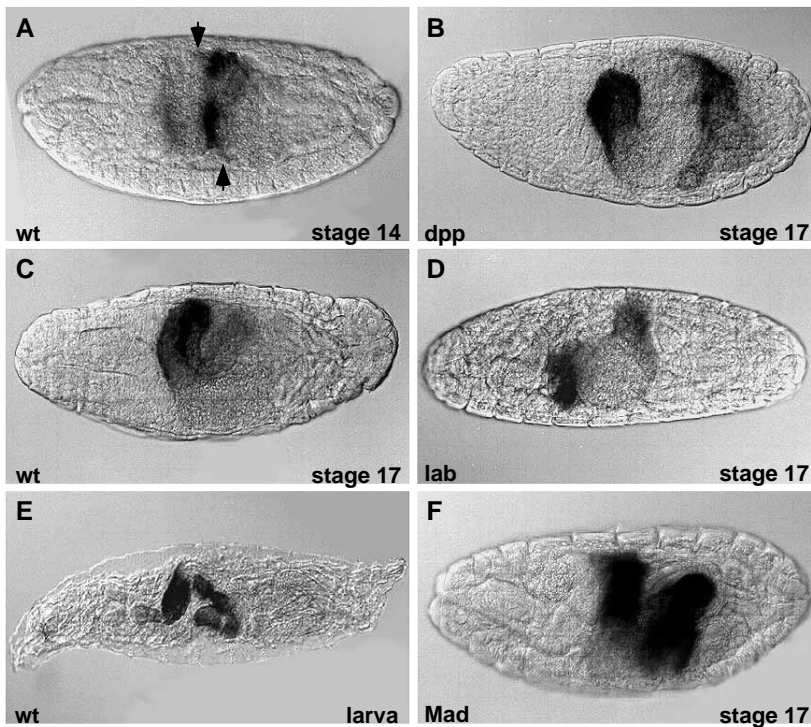


Fig. 4. Identification of a midgut endoderm enhancer active in *Mad* mutant embryos. (A,C,E) P{*mex1*βgal} wild-type expression; (B,D,F) P{*mex1*βgal} expression in *dpp*, *lab* and *Mad* mutant embryos, respectively. (A) Stage 14 embryo expressing β-galactosidase from the *mex1* enhancer in the midgut endoderm. The expression is strongest near the second midgut constriction but is also evident anterior and posterior to the constriction (indicated by arrowheads) in a broader band of expression than seen for *lab* (Chouinard and Kaufman, 1991). (C) A high level of expression, in a short stretch of midgut endoderm, is maintained in stage 17 embryos. (E) High levels of expression (still broader than *lab*) continue into the first larval instar. (B,D,F) Only the anterior and posterior ends of *mex1* expression are seen in the midgut endoderm of *dpp*, *lab* and *Mad* mutant stage 17 embryos, respectively.

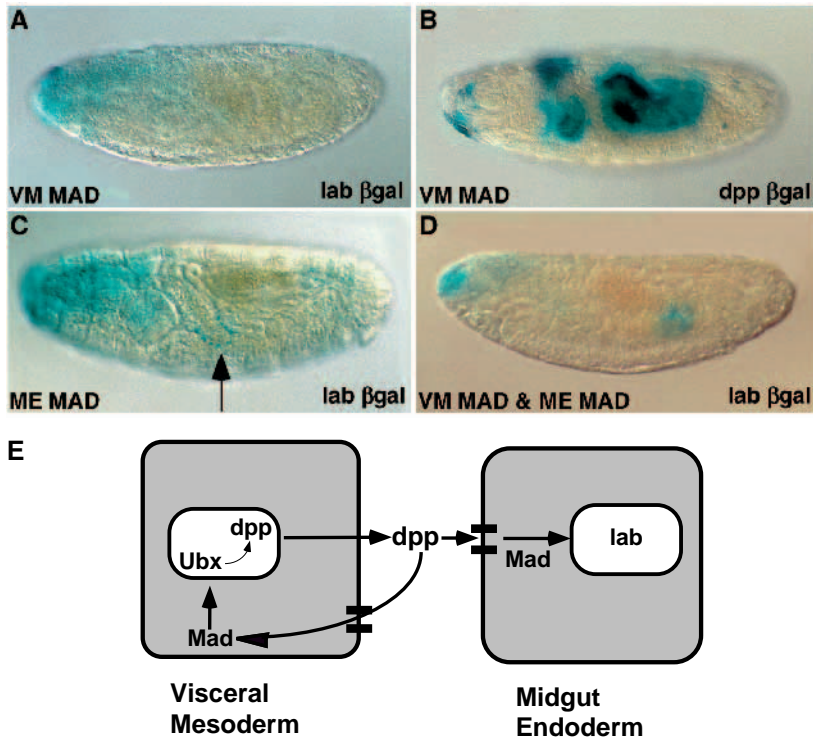


Fig. 5. MAD functions in DPP-responsive cells. (A,C,D) *lab* expression in *Mad* mutant embryos with tissue-specific expression of MAD and ubiquitous DPP; (A) MAD in the visceral mesoderm [VM], (C) MAD in the midgut endoderm [ME] and (D) MAD in the visceral mesoderm and midgut endoderm. (B) *dpp* expression in an embryo with MAD in the visceral mesoderm without ubiquitous DPP. Comparing A with B reveals that MAD in the visceral mesoderm cannot rescue *lab* expression in the midgut endoderm but rescues *dpp* maintenance in the visceral mesoderm. To evaluate the extent of *dpp* maintenance rescued, compare D to wild-type and homogeneous *Mad* mutant embryos in Fig. 2E,F. (C) MAD in the midgut endoderm rescues *lab* expression in that tissue; *lab*-expressing cells (identified by an arrow) are seen in the elongating midgut. (D) The rescue of *dpp* maintenance by MAD in the visceral mesoderm improves the rescue of *lab* by MAD in the midgut endoderm in an embryo expressing MAD in both tissues. (E) Schematic depiction of the results of the tissue-specific expression experiments.

Studies with anti-MAD serum on wild-type embryos revealed a ubiquitous tissue distribution (Fig. 6D) during embryonic development. This result is consistent with our RNA in situ hybridization experiments which demonstrated that *Mad* transcripts are ubiquitous in the embryo (Sekelsky et al., 1995). Fig. 6E shows a high magnification confocal micrograph of the midgut endoderm in ps7 of a wild-type stage 16 embryo indicating that MAD is cytoplasmic in this tissue. Further, the patterns of MAD expression and subcellular localization are unaltered in *dpp^{s4}* mutant embryos which specifically lack DPP expression in ps7 (data not shown). This cyto-

plasmic localization is consistent with MAD's participation in signal transduction in DPP-responsive cells.

Drosophila MAD functions with *Xenopus* BMP-4

The functional conservation and cellular and developmental similarities of DPP and BMP-2 and BMP-4 led us to determine if MAD's role in DPP signaling is conserved in vertebrates. We conducted a series of experiments using *Xenopus* animal pole explants (Fig. 7A). Different TGF- β family ligands can convert these explants, normally fated to form ectoderm, into one of two easily distinguished types of mesoderm, dorsal or

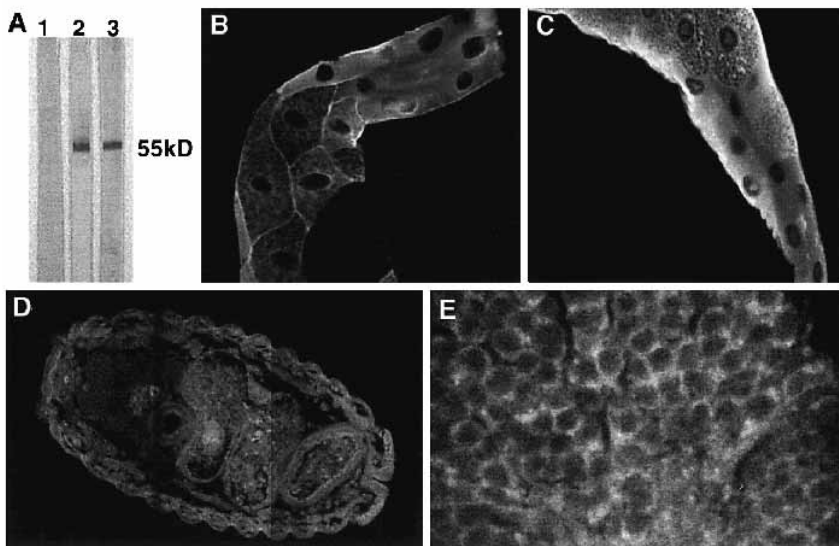


Fig. 6. MAD is a ubiquitously expressed cytoplasmic protein. (A) Three strips from a single western blot of protein extracted from *P{UmMad}* *lab* third instar larval salivary glands. Lane 1 was incubated with preimmune serum. Lane 2 was incubated with anti-MAD polyclonal serum. Lane 3 was incubated with anti-MYC monoclonal antibodies. The preimmune serum shows no reactivity while the anti-MAD and anti-MYC antibodies both recognize a $55 \times 10^3 M_r$ protein. (B) A salivary gland from a *P{UmMad}* *lab* third instar larva which was incubated with anti-MYC antibodies revealing a cytoplasmic subcellular localization for MAD. The cells nearest the salivary gland's common duct are on the upper right. (C) *P{UmMad}* *lab* third instar larval salivary gland incubated with anti-MAD serum which shows the same subcellular localization, though some staining in a few nuclei is seen. The cells nearest the salivary gland's common duct are at the lower right. (D) Composite confocal micrograph of a stage 16

wild-type embryo which was incubated with anti-MAD serum showing that MAD is ubiquitously expressed. (E) Confocal micrograph (1500 \times) of ps7 midgut endoderm, from a wild-type embryo of comparable stage to that shown in D. MAD appears to be a cytoplasmic protein in all tissues.

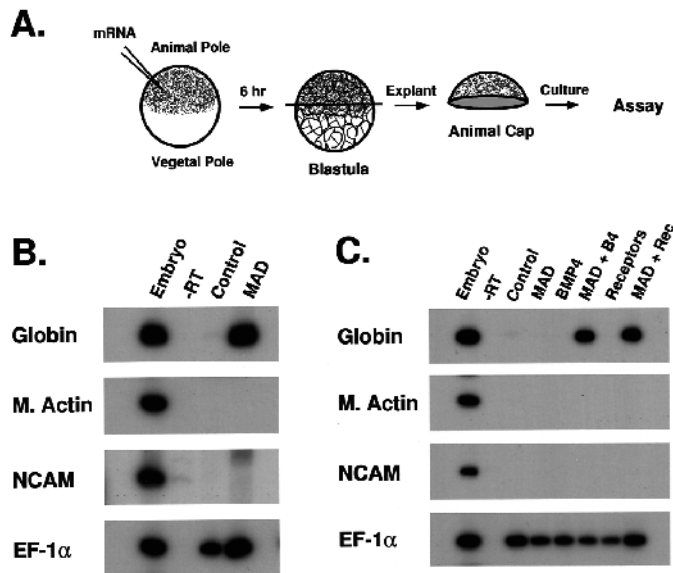


Fig. 7. *Drosophila* MAD induces ventral mesoderm in *Xenopus*. (A) Assay of MAD function in *Xenopus* animal caps. 1-cell embryos were injected at the animal pole. At the blastula stage, animal caps were explanted and cultured until sibling embryos developed to stage 35 (tadpole). (B) Autoradiograph of an assay from embryos injected with RNA encoding *Drosophila* MAD (2 ng). After injection, animal caps were dissected, cultured and total RNA harvested. The RNA was analyzed by RT-PCR for the presence of actin, globin, NCAM and EF-1 α transcripts. The lane marked E contains total RNA harvested from whole embryos as a positive control. The lane marked -RT, is identical to the E lane except that reverse transcriptase (RT) was not included as a negative control. The lane marked C corresponds to animal caps treated identically to other samples except no RNA was injected. EF-1 α is a ubiquitously expressed transcript (Krieg et al., 1989) and demonstrates that roughly equal amounts of RNA are included in each reaction. (C) Embryos were injected with mRNA that encodes MAD (100 pg), BMP-4 (250 pg), MAD (100 pg) + BMP-4 (250 pg), Receptors: BMP-4 type I receptor (10 pg) + activin type II receptor (10 pg), or MAD (100 pg) + Receptors (10 pg each). After injection embryos were treated as for B.

ventral (Klein and Melton, 1994). When mRNA encoding *Drosophila* MAD is injected into the animal pole, ectodermal explants are converted into ventral mesoderm as shown by the expression of globin mRNA (Fig. 7B). Dorsal mesoderm (muscle actin; Mohun et al., 1984) and neural (NCAM; Kintner and Melton, 1987) markers are not induced by MAD. This result mimics the effect of BMP-4, the vertebrate homolog of *dpp* (Jones et al., 1992; Dale et al., 1992).

Subsequently, we injected *Xenopus* BMP-4 or pooled *Xenopus* BMP-4 type I and activin type II receptors together with *Drosophila* MAD. Each of the injected mRNAs was of insufficient dosage to induce ventral mesoderm. Subthreshold doses of MAD, with either BMP-4 or the pooled receptors, acted synergistically to induce ventral mesoderm (Fig. 7C). These experiments suggest that MAD is a highly conserved element of the DPP/TGF- β signaling pathway.

DISCUSSION

A key to understanding the mechanisms by which TGF- β

signals elicit cellular and developmental responses is the ability to manipulate elements of the signaling pathway. Our approach relies on genetic screens for the identification of potential candidates and on further analyses to sift among these, pinpointing those most likely to act in the signal transduction cascade. The MAD protein is a prime candidate for such a signal transduction element.

Loss-of-function *Mad* mutant phenotypes are remarkably similar to *dpp* mutant phenotypes, including embryonic dorsal/ventral patterning and midgut defects as well as imaginal disk-derived adult appendage defects (Raftery et al., 1995; Sekelsky et al., 1995). Recently, a role for *dpp* in the developing eye has been described (reviewed in Heberlein and Moses, 1995). A clonal analysis of strong hypomorphic alleles of *Mad* in eye disks reveals that *Mad* mutant clones have the same effect on eye development as *dpp* mutant clones (Wiersdorff et al., 1996). This result reinforces the proposal that every event that requires *dpp* also requires *Mad*.

Our analysis of several reporter genes in *Mad* mutant backgrounds show conclusively that *Mad* is not involved in regulating *dpp* transcription. A function for *Mad* downstream of *dpp* transcription is demonstrated for DPP-dependent events in ps3 and ps7 of the embryonic midgut including the induction of *lab* in the endoderm, the maintenance of *dpp* and *Ubx* expression and the repression of *Scr* in the visceral mesoderm. The *Scr* results support the suggestion (Hurst et al., 1993) that a DPP-mediated autoregulatory loop exists in the visceral mesoderm of ps3 and ps7. The failure of MAD expression in the visceral mesoderm to rescue *lab* induction demonstrates that MAD is not required post-transcriptionally to generate the DPP signal. However, the restoration of *lab* induction when MAD is expressed only in the endoderm shows that MAD expression in cells receiving a DPP signal is sufficient to restore a DPP-dependent response.

Consistent with the absence of secretion or nuclear localization signal sequences (Sekelsky et al., 1995), our immunohistochemical studies indicate that MAD is a cytoplasmic protein whose subcellular distribution is unaltered in *dpp* mutant genotypes. Graff et al. (1996) detect both nuclear and cytosolic locations for MAD protein. However, their biochemical assay utilizes embryos which display a functional response to large amounts of injected MAD. Our immunohistochemical studies detect wild-type levels of MAD. The localization of MAD suggests a factor that is available to respond to the activation of DPP receptors. Taken together, the available data suggest that MAD participates in all DPP-dependent signal transduction events. The demonstration that *Drosophila* MAD functions synergistically with *Xenopus* BMP-4 to specifically induce ventral mesoderm suggests that MAD's role in DPP signal transduction is highly conserved.

Prior to this study, the only identified gene product that may contribute to the DPP signal transduction pathway is *schnurri* (*shn*, Arora et al., 1995; Grieder et al., 1995; Staehling-Hampton et al., 1995). However, it has yet to be established if all DPP-dependent events require *shn* function. For example, embryos with complete loss of *shn* maternal and zygotic function do not resemble *dpp* null embryos (Grieder et al., 1995).

Given that MAD appears to be a component of the DPP signal transduction pathway, several important questions are immediately raised. Is MAD a general factor involved in the signaling of all TGF- β family members in *Drosophila* or is

MAD specific to pathways induced by DPP-receptor activation? While no firm answer can be given, the overall concordance of *Mad* and *dpp* mutant phenotypes suggests that MAD may be specific to DPP signaling. Further, aspects of the phenotype elicited by mutations in 60A, another *Drosophila* TGF- β family member, are not shared by *Mad* mutants (K. Wharton, personal communication).

The multiplicity of *Mad*-like genes identified in several non-*Drosophila* species demonstrate that *Mad* is a member of a protein family and suggest that *Drosophila melanogaster* will also have multiple *Mad*-like genes. Results presented here and in Graff et al. (1996) indicate that members of this protein family participate in the interpretation of TGF- β signals. We propose the name DOT (Downstream of TGF- β) for this protein family.

Our primary interest now is to understand how these various DOT proteins contribute to TGF- β signaling. In *Xenopus*, two different DOT genes produce qualitatively distinct effects on mesoderm induction (Graff et al., 1996), suggesting that different DOT proteins contribute to different TGF- β signal transduction pathways. Whether the multiplicity of DOT proteins is fully explained as elements downstream of different receptors remains to be determined. Another critical issue in understanding DPP signaling is to elucidate how different levels of signal can produce different biological responses. A graded requirement for DPP has been demonstrated for dorsal-ventral patterning of the *Drosophila* embryo. Modulation of the level of that signal can lead to the establishment of different fates within the developing dorsal ectoderm (Ferguson and Anderson, 1992; Wharton et al., 1993). From a clonal analysis of *saxophone* mutations, we have suggestive evidence of a graded requirement for DPP in imaginal disk development as well (M. Singer and W. M. G., unpublished data). What is the contribution of MAD and its relatives to the amount and types of signals that are transduced? For example, different levels of DPP receptor activation might lead to quantitatively distinct levels of MAD activation, or alternatively to activation of different constellations of DOT proteins. It is critical to address these and other cellular aspects of MAD function in order to understand the contributions of TGF- β signaling to development.

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