

# The Zinc Finger Protein Schnurri Acts as a Smad Partner in Mediating the Transcriptional Response to Decapentaplegic

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In *Drosophila*, a BMP-related ligand Decapentaplegic (Dpp) is essential for cell fate specification during embryogenesis and in imaginal disc development. Dpp signaling culminates in the phosphorylation and nuclear translocation of Mothers against dpp (Mad), a receptor-specific Smad that can bind DNA and regulate the transcription of Dpp-responsive genes. Genetic analysis has implicated Schnurri (Shn), a zinc finger protein that shares homology with mammalian transcription factors, in the Dpp signal transduction pathway. However, a direct role for Shn in regulating the transcriptional response to Dpp has not been demonstrated. In this study we show that Shn acts as a DNA-binding Mad cofactor in the nuclear response to Dpp. Shn can bind DNA in a sequence-specific manner and recognizes sites within a well-characterized Dpp-responsive promoter element, the B enhancer of the *Ultrabithorax* (*Ubx*) gene. The Shn-binding sites are relevant for *in vivo* expression, since mutations in these sites affect the ability of the enhancer to respond to Dpp. Furthermore we find that Shn and Mad can interact directly through discrete domains. To examine the relative contribution of the two proteins in the regulation of endogenous Dpp target genes we developed a cell culture assay and show that Shn and Mad act synergistically to induce transcription. Our results suggest that cooperative interactions between these two transcription factors could play an important role in the regulation of Dpp target genes. This is the first evidence that Dpp/BMP signaling in flies requires the direct interaction of Mad with a partner transcription factor. © 2000 Academic Press

**Key Words:** *Drosophila*; BMP; *Ultrabithorax*.

## INTRODUCTION

Secreted ligands belonging to the transforming growth factor- $\beta$  (TGF- $\beta$ ) superfamily play critical roles in a variety of biological functions, including regulation of the cell cycle, endocrine function, cell proliferation, and differentiation. The bone morphogenetic proteins (BMPs) that make up the largest subgroup of the TGF- $\beta$  superfamily are involved in regulating growth and embryonic development in diverse organisms including nematodes, flies, frogs, mice, and humans (Massagué, 1998; Raftery and Suther-

land, 1999; Wisotzkey *et al.*, 1998). The broad range of activities attributed to the TGF- $\beta$ /BMP ligands has spurred interest in understanding the molecular mechanisms by which the diverse cellular responses to these growth factors are mediated. In *Drosophila* three BMP-related ligands, *dpp*, *screw* (*scw*), and *glass bottom boat*, have been identified that affect embryonic patterning (Arora *et al.*, 1994; Padgett *et al.*, 1987; Wharton *et al.*, 1991). Of these, the role of *dpp* has been most extensively studied. Dpp is required at multiple times during embryogenesis, as well as in the specification of adult structures (Spencer *et al.*, 1982). In the early embryo, Dpp acts combinatorially with Scw to specify cell fate in the entire dorsal region, while at later stages of embryogenesis *dpp* is involved in more localized patterning events, such as dorsal closure, tracheal and gut develop-

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ment, and differentiation of the somatic and visceral mesoderm (Ferguson and Anderson, 1992; Frasch, 1995; Neul and Ferguson, 1998; Nguyen et al., 1998; Staehling-Hampton et al., 1994; Wharton et al., 1993). During larval development Dpp is involved in patterning the imaginal discs and displays many of the characteristics of a morphogen in specification of the wing disc (Neumann and Cohen, 1997).

Ligands belonging to the TGF- $\beta$  superfamily share common features in their mechanism of signal transduction. Like other members of this group, BMPs signal through a heteromeric complex of type I and type II receptor serine-threonine kinases. Formation of the ligand-receptor complex allows the type II receptor to phosphorylate the type I receptor. Upon activation, the type I receptor mediates intracellular signaling by phosphorylating a receptor-specific member of the Smad family of proteins within a carboxy-terminal SXS motif. In vertebrates, three distinct Smads (Smad1, 5, and 8) are activated in response to BMP ligands, while Smad2 and Smad3 are implicated in responses to TGF- $\beta$  and activin. Following phosphorylation, the receptor-specific Smads associate with a common Smad (Smad4) and the resulting complex translocates from the cytoplasm into the nucleus. The strength and duration of TGF- $\beta$  signaling are modulated by a third subgroup of inhibitory Smads that act as antagonists of receptor signaling (reviewed in Derynck and Feng, 1997; Heldin et al., 1997; Massagué, 1998). In *Drosophila*, genetic and biochemical studies have established that a single type II receptor Punt (Put) and two type I receptors, Thick veins (Tkv) and Saxophone (Sax), are involved in mediating Dpp signaling (Brummel et al., 1994; Letsou et al., 1995; Nellen et al., 1996; Neul and Ferguson, 1998; Nguyen et al., 1998; Penton et al., 1994; Ruberte et al., 1995; Xie et al., 1994). The Tkv receptor has been shown to phosphorylate Mad, the fly ortholog of Smad1, resulting in its translocation into the nucleus along with the Smad4 ortholog Medea (Das et al., 1998; Hoodless et al., 1996; Hudson et al., 1998; Newfeld et al., 1996; Raftery et al., 1995; Sekelsky et al., 1995; Wisotzkey et al., 1998).

The signal-dependent translocation of a heteromeric Smad complex to the nucleus is a key regulatory event in TGF- $\beta$ /BMP signal transduction. Smads have DNA-binding activity and are thought to regulate the expression of downstream target genes in combination with tissue-specific transcription factors (Derynck and Feng, 1997; Heldin et al., 1997; Massagué, 1998; Whitman, 1998). Since Smads recognize DNA sequences of low complexity, it is believed that interactions with other transcription factors are necessary to provide specificity in the response to the signal, and allow different cellular responses to be elicited by the limited number of Smads identified. In addition, Smads bind DNA with low affinity, so interaction with partner DNA-binding proteins could increase the range and sensitivity of the response to the ligand. A paradigmatic example is the formation of a complex containing Smad2/4 and the forkhead domain protein FAST-1, that mediates the activation of several TGF- $\beta$  and activin-responsive genes

(Chen et al., 1996; reviewed in Whitman, 1998). Smad proteins can also act synergistically with transcription factors that bind DNA independently, as exemplified by the regulation of the *tinman* (*tin*) gene in the *Drosophila* mesoderm (Xu et al., 1998). Recent studies have shown that Smad activity can be modulated by association with coactivators or repressors that do not bind DNA, such as the CREB-binding protein (CBP), transcription factor  $\mu$ E3 (TFE3), and Melanocyte-specific gene-1 (MSG) (reviewed in Massagué and Wotton, 2000). While several Smad-interacting DNA-binding proteins are known in the activin and TGF- $\beta$  pathways, the vertebrate zinc finger protein OAZ is the only transcription factor to be identified in BMP signaling thus far (Hata et al., 2000).

In *Drosophila*, promoter analyses of the *dpp* target genes *vestigial* (*vg*), *tin*, and *Ubx* have identified Mad- and Medea-binding sites that are essential for expression *in vivo* (Kim et al., 1997; Waltzer and Bienz, 1999; Xu et al., 1998). However transcriptional partners that act with *Drosophila* Smads to mediate the response to Dpp remain to be characterized. Here we present evidence that the *shn* gene is likely to play such a role. Mutations in *shn* cause embryonic lethality and result in a dorsal open phenotype remarkably similar to that caused by zygotic loss of the Dpp receptors *tkv* and *put* (Nüsslein-Volhard et al., 1984). In addition mutations in *shn* enhance the phenotype of partial loss-of-function mutations in *dpp*, suggesting that these genes are linked functionally. *shn* encodes a protein with multiple zinc finger DNA-binding domains, that is structurally related to human MBP-1/PRDII-BF1 and MBP-2 (Arora et al., 1995; Grieder et al., 1995; Staehling-Hampton et al., 1995). Analysis of gene expression in *shn* mutant embryos indicates that *shn* activity is required for the transcription of *dpp*-responsive genes in multiple tissues and at different developmental stages. These genes include *pannier* in the dorsal ectoderm, *tin* in the dorsal mesoderm, and *wingless* (*wg*) and *labial* (*lab*) in the developing gut (Arora et al., 1995; Grieder et al., 1995; Staehling-Hampton et al., 1995). The observation that ubiquitous expression of the ligand cannot restore expression of *lab* in a *shn* mutant embryo indicated that Shn acts downstream of Dpp signaling (Grieder et al., 1995). Based on these genetic studies it was proposed that Shn is involved in regulating the transcription of Dpp-responsive genes. Although these data implicate Shn in Dpp signaling, the mechanism by which Shn functions has not been determined thus far.

At least four simple models for *shn* function are compatible with the above observations. The first possibility is that Shn and Mad independently regulate target gene expression by binding DNA. In the second alternative, Shn could act indirectly, either by regulating an activator of Dpp target genes or by inhibiting a negative regulator of Dpp signaling. A third model is that Shn does not bind DNA but interacts with Mad to regulate target gene expression, analogous to the manner in which the non-DNA-binding Smad partners MSG, TFE3, and CBP function. Finally, it is also possible that Shn regulates transcription in concert

with Mad, by acting as a FAST-1-like cofactor. In order to distinguish between these alternatives, we examined whether Shn can interact directly with Mad, and if Shn can bind DNA at *in vivo* relevant sites in Dpp-responsive promoter elements. In this study we provide evidence in favor of the fourth model. We demonstrate a direct physical interaction between Shn and Mad using yeast two-hybrid and coimmunoprecipitation assays. We use DNase footprint analysis to show that Shn binds the Ubx B midgut enhancer, a well-characterized promoter element that mediates the Dpp-dependent transcriptional response in the embryonic visceral mesoderm. In order to determine the relative contributions of DNA binding and protein-protein interaction, we have developed a cell culture assay for Dpp signaling using the Ubx B reporter. We find that both functions contribute to the ability of Shn and Mad to activate transcription from this promoter in a synergistic manner. Our data provide the first evidence that Shn acts as a transcriptional partner of Mad in mediating the response to Dpp signaling.

## MATERIALS AND METHODS

### Plasmid Construction

The full-length *shn* cDNA was cloned as a *Bgl*III-*Sma*I fragment into the *Bam*HI and *Hinc*II sites of pCITE4b (Novagen), by introducing a unique *Bgl*III site 10 bp upstream of the ATG. For mapping interaction domains Shn fragments were subcloned into the appropriate pCITE vector to maintain an open-reading frame using endogenous restriction sites corresponding to the amino acid residues in parentheses: *Eco*RI (341), *Nhe*I (687), *Bam*HI (968), *Eco*RI (1069), *Hinc*II (1328), *Bam*HI (1441), *Sal*I (1463), *Cla*I (1635), *Nco*I (1676), *Eco*RI (1776), *Nco*I (1981), *Sal*I (2318), *Nco*I (2342), *Not*I (2529) in the vector. The pCITE vectors are optimized for protein expression in reticulocyte systems. Mutations in the S1 and S2 Shn-binding sites in Ubx B were generated using a PCR-based strategy that introduced a 5' *Bgl*III site. A *Bgl*III-*Xba*I fragment was inserted into pGL2p-luc (Promega) to generate BS1S2-luc, and into the C4PLZ (S. Crews) to construct the BS1S2-LacZ reporter. In BS1S2 the wild-type sequences TCAGGGGGAGCCA and CGGGTGCACCC were replaced by CTAATATTGACTAA and CATGTACATAT, respectively. A fragment from BM2 containing mutant Mad sites (Thuringer *et al.*, 1993) was substituted into BS1S2 to generate BM2S1S2-luc. pCMV5b-Mad-Flag and pCMV5b-TkvA-HA (Hoodless *et al.*, 1996), pCMV5-Medea (Wisotzkey *et al.*, 1998), pCMV5-Babo-HA, and pCMV5-dSmad2-Flag (Brummel *et al.*, 1999) have been previously described.

### Yeast Two-Hybrid Assays

Tests for interaction in yeast were carried out as described in (Gyuris *et al.*, 1993). Shn bait was generated by blunting a *Bgl*III-*Xba*I fragment containing full-length Shn into the *Bam*HI and *Xho*I sites of pEG202. Prey plasmids Mad FL, Mad MH2+L, and Mad MH1 were generated by subcloning an *Xho*I fragment, an *Eco*RI and *Xho*I fragment, and an *Eco*RI fragment from pEG202 Mad (L. Raftery, unpublished) into pJG4-5, respectively.

### Immunoprecipitation and Immunoblotting

COS-1 cells maintained in DMEM with 10% FBS were transiently transfected with the indicated constructs using Lipofectamine (Life Technologies). For coimmunoprecipitation, cells were lysed at 36 h posttransfection and incubated with anti-Flag M2 monoclonal antibody (IBI, Eastman Kodak), followed by adsorption to protein G-Sepharose (Pharmacia; Hoodless *et al.*, 1996). Precipitates were washed and resolved on SDS-PAGE.

### GST Pull-Down Assays

Full-length GST-Mad is a *Bam*HI/*Not*I fragment from pJGMad blunted into *Bam*HI and *Sma*I sites in pGEX3. GST-Mad FL was digested with *Eco*RI and recircularized to form GST-Mad MH2+L. GST-Shn 1441-1776 is a *Bam*HI-*Eco*RI fragment inserted into pGEX 4T-1. pCITE Mad was generated by digesting pEG202 Mad with *Bam*HI and *Not*I and subcloning into pCITE4c. GST-fusion proteins were expressed in the protease-deficient *Escherichia coli* strain BL21(DE3). Following cell lysis, fusion proteins were bound to glutathione agarose beads and preincubated in binding buffer for 2 h at room temperature prior to the addition of *in vitro* translated <sup>35</sup>S-labeled polypeptides (TNT, Promega). Binding reactions were carried out overnight at 4°C in 20 mM Hepes, pH 7.9, 100 mM KCl, 100 mM NaCl, 5 mM MgCl<sub>2</sub>, 1 mM DTT, 0.5% Triton, 0.5% nonfat milk, 0.5% BSA, 20% glycerol, followed by four washes with 10 mM Tris, pH 8.0, 200 mM NaCl, 1 mM EDTA, 0.5% Triton. Bound proteins were boiled in loading buffer, resolved on SDS-PAGE, and visualized by fluorography or phosphorimaging.

### Gel-Shift and Site-Selection Assays

Shn DBD1 (residues 340-621) and DBD2 (1675-1980) were expressed as His-tagged proteins using pRSET vectors (Invitrogen). Soluble DBD1 and DBD2 polypeptides were purified on a Ni-NTA matrix in the presence of 1 mM PMSF, 1 mM benzamidine, and 10 μM ZnCl<sub>2</sub> and eluted with 200 mM imidazole prior to dialysis against buffer Z (Hoey *et al.*, 1988). For gel-shift assays, purified protein was incubated at 4°C with <sup>32</sup>P-labeled double-stranded oligo containing the MBP-1 (ACATTTGGGGAATCCCCTTA-ATT) site in DNA-binding buffer (110 mM KCl, 10 mM MgCl<sub>2</sub>, 25 mM Tris, pH 6.9, 1 mM β-mercaptoethanol, 10 μM ZnCl<sub>2</sub>, 0.05% NP-40, 20% glycerol). Electrophoresis was carried out on a 5% native polyacrylamide gel (0.5× TBE, acrylamide:bisacrylamide 29:1). Site selection was performed by gel shifting the SELEX oligo (5'-GTA AACGACGGCCAGTGT TTTNNNNNNNNNNNNNNNNNNNN- TTTGTCGACCATGGTCATAGCTGTTTCC-3') using the conditions described. The region of the gel predicted to contain the shifted band was cut out and the eluted DNA was PCR-amplified using M13 primers to provide substrate for the next round of site selection. Following three rounds of binding and amplification, the oligos were cloned and sequenced.

### DNaseI Footprint Analysis

GST-Shn DBD1 (residues 340-621) and GST-Shn DBD2 (residues 1675-1897) were used in footprint experiments as they were more soluble than the His-tagged forms. GST-fusion proteins were purified as described except that 1 mM PMSF, 1 mM benzamidine, and 10 μM ZnCl<sub>2</sub> were added to all solutions. Purified protein was flash frozen and stored at -70°C. Footprinting reactions were carried out as described previously, except that probes were prepared by PCR amplification using <sup>32</sup>P-labeled kinased oligos (Hoey

et al., 1988). M13 forward and reverse primers were used to amplify a sequence containing the MBP probe cloned into pBluescript. Probes for Ubx B were generated using M13 primers and an internal primer (5'-GATGTTTCTGGACTGGCG-3'). Asymmetrically labeled PCR products were gel-purified prior to use. DNase footprint analysis was carried out to confirm that the mutant sites in Ubx BS1S2 no longer bind Shn.

### **Fly Strains and Phenotypic Analysis**

Reporter lines bearing Ubx B (Bhz) were obtained from M. Bienz (Thuringer et al., 1993). Multiple independent transgenic lines bearing the BS1S2-LacZ reporter were generated by germline transformation. Transgenic lines mutant for the Shn S1 site and deleted for S2 were generated in a similar manner. Misexpression of *dpp* was carried out using a heat shock-inducible transgene P[hs-*dpp*.BP] (Twombly et al., 1996). The *shn*<sup>P4738</sup> allele used in this study is an embryonic transcriptional null (Arora et al., 1995). Homozygous mutant embryos were recognized by the absence of a *wg*-LacZ reporter in the *CyO* balancer. For analysis of the Ubx B reporter and its derivatives, 0–15 h egg collections at 25°C were processed for staining as described below. For heat shock induction of *dpp*, a 0- to 15-h egg collection at 25°C was subjected to a 1-h heat shock at 37°C, allowed to recover for 1 h at 25°C, followed by a second heat shock, and fixed after a 3-h recovery period (modified from Newfeld et al., 1996). For immunohistochemistry, embryos were dechorionated and fixed in 4% formaldehyde according to standard protocols. LacZ expression was detected by incubation with a mouse monoclonal anti-LacZ antibody followed by an alkaline phosphatase-conjugated secondary antibody (Promega) as described in (Nguyen et al., 1998). Stained embryos were dehydrated in ethanol, rehydrated in PBT, and mounted in 70% glycerol:30%PBT.

### **Luciferase Reporter Assays**

C3H10T1/2 fibroblast cells (ATCC CCL-226) were transiently transfected with the reporter plasmid, pTKβ-gal, and the indicated constructs, or with vector alone to maintain a constant amount of total DNA. Cells were seeded at a density of  $1.2 \times 10^4$  cells in 12-well plates and grown to 70% confluence, in DMEM supplemented with 10% FBS and nonessential amino acids. Cells were transfected with a total of 1.5 μg DNA per well, as described above. Cells were harvested and lysed 36 h posttransfection. Luciferase activity was measured using the Luciferase assay system (Promega) and normalized with respect to β-galactosidase activity using the Galacton-Plus system (Tropix Inc.).

## **RESULTS**

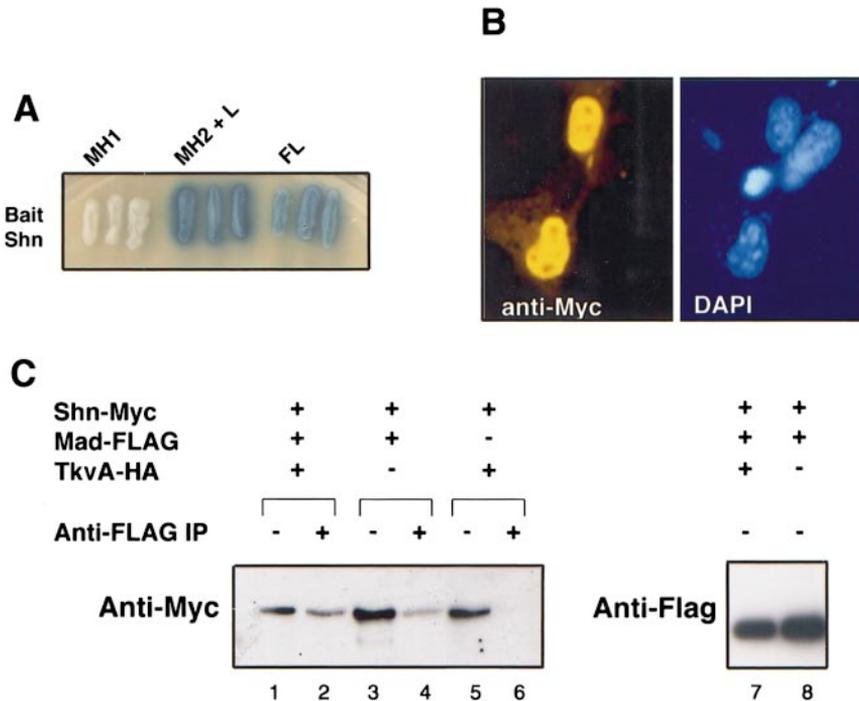
### **Shn Interacts with Mad in Yeast Two-Hybrid Assays**

Genetic studies have suggested that Shn is likely to act as a nuclear effector in the Dpp signaling pathway (Arora et al., 1995; Grieder et al., 1995; Staehling-Hampton et al., 1995). The transcriptional response to TGF-β and activin signaling is known to require the association of Smad proteins with other transcription factors. To determine whether Shn acts in an analogous manner we decided to test for interactions between Shn and Mad, the Drosophila

Smad implicated in Dpp signaling. We initially assayed whether the two proteins interact directly using the yeast two-hybrid assay (Gyuris et al., 1993). A cDNA clone encoding full-length Shn was fused to the heterologous LexA DNA-binding domain and used as a bait. Smads characteristically contain an amino-terminal Mad homology region 1 (MH1) and a carboxy-terminal Mad homology region 2 (MH2) separated by a poorly conserved linker region (Hoodless et al., 1996; Savage et al., 1996). Prey plasmids were generated that expressed full-length Mad (FL), Mad MH1, or the MH2 domain along with the linker (MH2+L). In the two-hybrid assay, interaction of the bait and prey allows yeast expressing both proteins to grow on selective media and activate transcription of a β-galactosidase reporter. Based on both these criteria, we observed that Shn associated with Mad FL and Mad MH2+L, but did not interact with Mad MH1 (Fig. 1A). The failure of Mad MH1 to interact with Shn cannot be ascribed to lower levels of expression of this domain, since antisera directed against an HA epitope present in each prey fusion detected comparable levels of all three Mad polypeptides on Western blots (data not shown). Finally, none of the Mad prey proteins interacted with a bait plasmid containing the Drosophila homeodomain transcription factor Bicoid, indicating that the interaction between Mad and Shn is specific (data not shown).

### **Shn-Mad Interactions Can Be Detected in Cultured Cells**

In order to determine whether the interaction we detected between Shn and Mad in yeast also occurs *in vivo*, we assayed their association in COS cells using epitope-tagged proteins. Full-length Myc-tagged Shn showed tight nuclear localization in transfected cells, consistent with the observation that Shn is localized to the nucleus in Drosophila embryos (Fig. 1B; Staehling-Hampton et al., 1995). In the initial experiments, COS cells were cotransfected with Shn-Myc, Mad-Flag, and a HA-tagged constitutively activated form of Tkv (TkvA), the Dpp type I receptor. TkvA mimics signaling by phosphorylating Mad and causing its translocation into the nucleus in a ligand-independent manner (Hoodless et al., 1996). Cell lysates were immunoprecipitated with anti-Flag antisera and analyzed on Western blots probed to detect Shn. An aliquot of the sample prior to immunoprecipitation was loaded to test for levels of Shn or Mad expression. Anti-Myc antisera detected a ~280-kDa band corresponding to full-length Shn in the presence of TkvA, confirming that Mad and Shn can interact directly (Fig. 1C, lanes 1, 2). We then examined whether the Shn-Mad interaction was dependent on Dpp signaling by cotransfecting cells with Shn and Mad alone. As seen in lanes 3 and 4, Shn coimmunoprecipitated with Mad even in the absence of TkvA, although at slightly reduced levels (Fig. 1C). Previous studies have shown that even when Mad is transfected alone (i.e., in the absence of TkvA) it is evenly distributed between the nucleus and the cytoplasm. This may result from overexpression or basal BMP signaling in COS cells that causes partial phosphorylation and nuclear



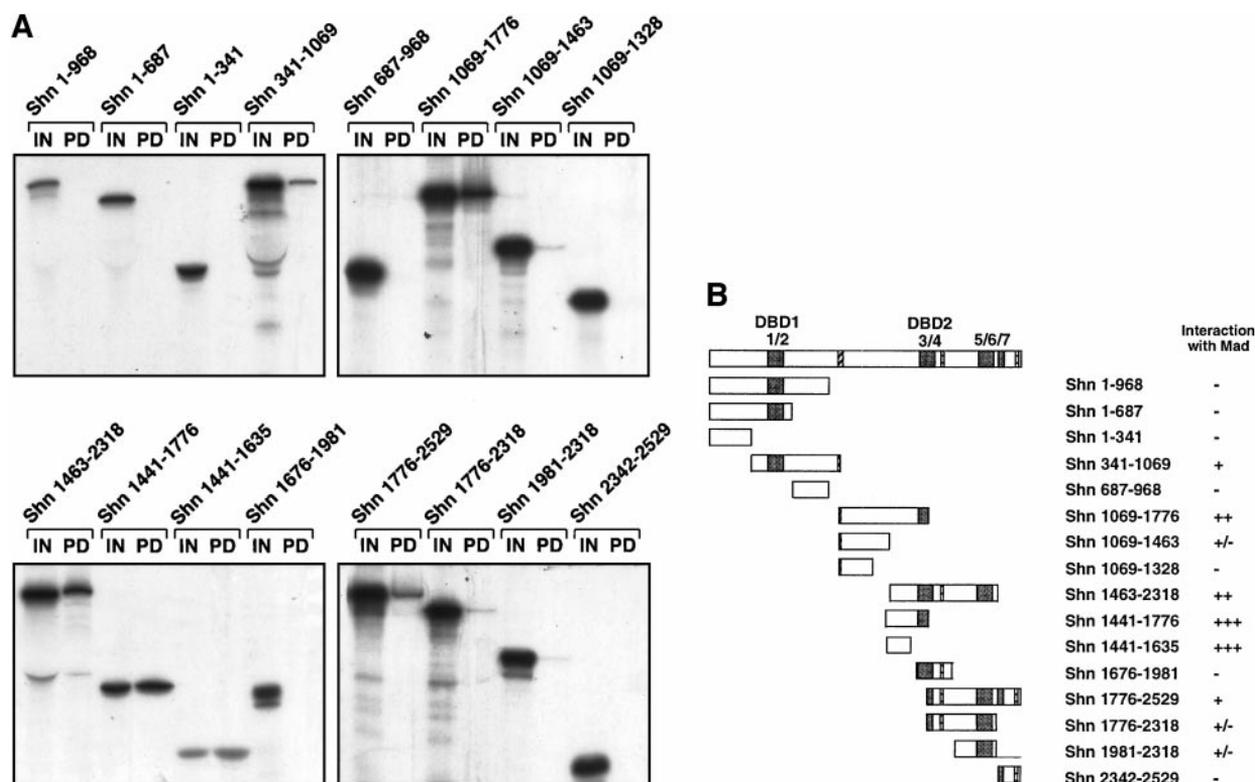
**FIG. 1.** Interaction between Shn and Mad in yeast and COS cells. (A) Functional interaction and *trans*-activation in yeast two-hybrid assays. Yeast were transformed with Shn bait (full-length Shn-LexA fusion protein) and different prey plasmids containing either Mad homology region 1 (MH1), Mad homology region 2 with the linker region (MH2+L), or full-length Mad (FL). Protein-protein interactions between Shn and Mad were detected by activation of the  $\beta$ -galactosidase reporter. Shn interacts specifically with the MH2+L region of Mad, and less strongly with Mad FL. No  $\beta$ -galactosidase activity was detected with MH1 or on control glucose plates. (B) Subcellular localization of Shn. COS cells transfected with Shn-Myc show accumulation in the nucleus, visualized by immunofluorescence using anti-Myc antibody and Cy3-conjugated secondary. Cells were counterstained with DAPI to visualize the nuclei. (C) Shn interacts with Mad *in vivo*. COS cells were transiently transfected with vectors carrying Shn-Myc, Mad-Flag, and TkvA-HA, as indicated. Cell lysates were subjected to immunoprecipitation (IP) with anti-Flag M2 antibody and then immunoblotted using anti-Myc antibody. Levels of Shn expression were monitored by loading an aliquot of the cell lysate prior to immunoprecipitation, in alternate lanes. Mad expression was monitored by probing a separate blot with anti-Flag antibody. Shn associates with Mad in the presence of TkvA, although an interaction is seen in a signaling-independent manner as well.

translocation of Mad (see Discussion; Hoodless *et al.*, 1996; Wisotzkey *et al.*, 1998). The presence of nuclear Mad could account for the significant interaction we see between Mad and Shn under these conditions. The failure to immunoprecipitate Shn in the absence of Mad demonstrates the specificity of the interaction (Fig. 1C, lanes 5, 6).

### Discrete Regions Mediate Shn-Mad Interactions

In order to delineate the domains in Shn that interact with Mad, we used GST pull-down assays. Since experiments in yeast indicated that the Mad MH2+L domain is sufficient for interaction with Shn, we expressed this region as a glutathione *S*-transferase (GST) fusion protein. Sixteen overlapping subclones encompassing the entire Shn coding region were used to generate [<sup>35</sup>S]methionine-labeled protein fragments using coupled *in vitro* transcription and translation (Fig. 2). The GST-Mad affinity matrix was tested for its ability to retain the <sup>35</sup>S-labeled Shn peptides. As

shown in Fig. 2A, Mad MH2+L interacts most strongly with two overlapping regions, Shn 1069–1776 and Shn 1463–2318. Experiments with full-length Mad yielded essentially similar results (data not shown). Further deletions allowed us to narrow the interaction to Shn 1441–1635, a region of 194 residues preceding the second set of paired zinc fingers (Figs. 2A, B). Two additional nonoverlapping fragments, Shn 341–1069 and Shn 1776–2529, showed a moderate interaction with Mad. Subdivision of these fragments significantly reduced their association with Mad. In control experiments Shn fragments failed to bind to a GST matrix or to the Mad MH1 domain, confirming the specificity of the interactions (data not shown). Thus we have identified a total of one strong and two weaker Mad interaction domains (MIDs) in Shn (Fig. 2B). We also found that Shn 1441–1776 showed strong binding to itself with an affinity comparable to that displayed for Mad MH2+L (data not shown). In addition we detected significant binding to Shn 341–1069 and Shn 1776–2529, fragments that contain



**FIG. 2.** Mapping of Shn and Mad interaction domains by *in vitro* pull-down assays. (A) Different fragments of Shn were tested for their ability to bind GST-Mad-fusion protein (Mad MH2+L). Equivalent amounts of the GST-fusion affinity matrix were incubated with *in vitro* translated [<sup>35</sup>S]methionine-labeled Shn polypeptides as indicated, and the bound protein (PD) was visualized by autoradiography. Of the *in vitro* translation reaction 10% was loaded in the input (IN) lanes. In control reactions Shn fragments were tested for binding to GST alone (not shown). Mad interaction can be localized to three nonoverlapping segments, Shn 341–1069, Shn 1441–1635, and Shn 1776–2529. Shn 1441–1776 shows the strongest binding to Mad. (B) Structural organization of Shn and schematic representation of the protein fragments used to map Mad interaction domains. The filled boxes represent the seven conserved C<sub>2</sub>H<sub>2</sub> zinc finger domains, the cross-hatched box marks the single C<sub>2</sub>HC finger, while the horizontal hatched bars indicate potential acidic activation domains. The column at right summarizes the interaction data obtained in the GST pull-down assays with Mad.

the other MIDs. The binding of Shn 1441–1776 is specific since it failed to bind either Shn 1–968 (a fragment that lacks a MID) or an unrelated protein (luciferase) (data not shown). Thus our results suggest that regions of the protein that are involved in association with Mad may also mediate homomeric Shn interactions.

### ***Shn Can Bind DNA in a Sequence Specific Manner***

As a prerequisite to determining whether *shn* can directly regulate the transcription of Dpp target genes, we assayed the ability of bacterially produced Shn to bind DNA. Shn contains a total of seven Cys<sub>2</sub>His<sub>2</sub> zinc finger motifs organized as two widely separated pairs and a triad of fingers at the carboxy-terminal (see Fig. 2B). The first two sets of fingers share 67 and 78% identity, respectively, with the corresponding paired finger domains in the human MBP-1 and MBP-2 proteins (Arora *et al.*, 1995). The carboxy-terminal triad of fingers is not represented in vertebrate homologs of Shn and has limited identity with

known zinc finger proteins. In the human protein both sets of paired fingers can bind DNA independently and recognize related but distinct sequence motifs (Fan and Maniatis, 1990; van't Veer *et al.*, 1992). Therefore we characterized the binding specificity of the two paired DNA-binding domains (Shn DBD1 and Shn DBD2) individually, rather than as part of a single protein.

Shn DBD1 and DBD2 were expressed as fusion proteins with an amino-terminal His tag. We used a PCR-based binding site selection assay to determine the optimal binding sites recognized by each set of Shn zinc finger domains (see Materials and Methods). DBD1 and DBD2 proteins were purified on Nickel affinity columns and mixed with double-stranded oligonucleotides containing a core region of 14 randomized bases flanked on either side by known sequence. A region of the gel predicted to contain the protein DNA complexes was excised and subjected to PCR amplification. After three successive cycles of binding, elution, and amplification, the oligos were cloned and sequenced. Sequence data from a set of 16 clones bound by

First pair of C <sub>2</sub> H <sub>2</sub> zinc fingers												
Position	1	2	3	4	5	6	7	8	9	10	11	12
G	100	100	80	0	0	0	100	7	0	0	0	0
A	0	0	0	100	47	0	0	0	0	0	0	7
T	0	0	20	0	40	7	0	93	93	0	0	13
C	0	0	0	0	13	93	0	0	7	100	100	80
Consensus	G	G	G	A	A/T	C	G	T	T	C	C	C

Second pair of C <sub>2</sub> H <sub>2</sub> zinc fingers											
Position	1	2	3	4	5	6	7	8	9	10	11
G	83	94	100	100	0	0	6	6	0	0	0
A	11	6	0	0	89	56	39	17	0	0	17
T	0	0	0	0	0	11	44	77	0	6	6
C	6	0	0	0	11	33	11	0	100	94	77
Consensus	G	G	G	G	A	A/C	A/T	T	C	C	C

**FIG. 3.** Consensus Shn-binding sites. A PCR-based site selection assay was used to determine the optimal binding sites for His-tagged Shn polypeptides corresponding to the first and second set of paired zinc finger DNA-binding domains. A total of 16 clones were analyzed for Shn DBD1, and 18 clones for DBD2. Numbers represent percentage occurrence of the indicated nucleotide at each position. Oligonucleotide sequences were aligned to generate the consensus binding sites for the respective Shn DNA-binding domains.

Shn DBD1 revealed that each clone contained a related 12-bp sequence 5'-GGGA(A/T)CGTTCCC-3' or its complement (Fig. 3). The consensus sequence is essentially palindromic in nature and consists of a central 6-nucleotide core flanked by GGG at the 5' location and CCC at the 3' end. Analysis of the sequences bound by Shn DBD2 identified an optimal binding site 5'-GGGGA(A/C)(A/T)TCCC-3' closely related to that recognized by Shn DBD1. One difference between the two consensus sequences is the spacing between the flanking GGG(N<sub>n</sub>)CCC bases, with  $n = 6$  for DBD1 and  $n = 5$  for DBD2.

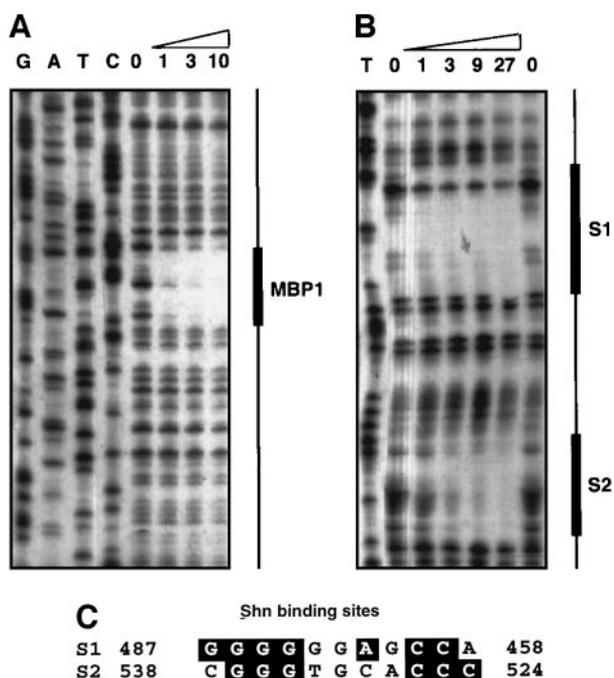
The optimal Shn-binding sites are similar to those recognized by its vertebrate orthologs. We therefore tested the bacterially produced proteins for their ability to recognize the sequence bound by human MBP-1 (Baldwin *et al.*, 1990; Fan and Maniatis, 1990; Rustgi *et al.*, 1990). Affinity-purified Shn DBD1 and DBD2 specifically bound oligos containing the MBP-1 site (GGGGATTCCCC) in gel-shift assays. This binding was resistant to the addition of 100-fold excess of nonspecific competitor DNA, but only a 10-fold excess of specific competitor prevented the formation of a DNA protein complex (data not shown).

### The Dpp-Responsive Ubx Enhancer Contains Shn-Binding Sites

We next wished to assay whether Shn recognizes sites in the promoters of genes that are induced by Dpp signaling in the embryo. Genetic studies have identified several genes that are candidates for direct regulation by *shn* based on the loss of their expression in both *dpp* and *shn* mutant em-

bryos (Arora *et al.*, 1995; Grieder *et al.*, 1995). Among these, the gut-specific expression of the *Ubx* gene is one of the most extensively characterized Dpp responses. The *Ubx* B element, a discrete 269-bp enhancer from the *Ubx* promoter, mediates *dpp*- and *wg*-dependent reporter gene expression in the midgut visceral mesoderm (Thuringer *et al.*, 1993). Recent studies have identified two Mad-binding sites within *Ubx* B. Mutations in these sites significantly reduced (but did not eliminate) the ability of the enhancer to respond to Dpp, indicating that they are required *in vivo* (Kim *et al.*, 1996; Szuts *et al.*, 1998).

In order to determine whether *shn* also plays a role in *Ubx* B transcription, we examined the enhancer for the presence of Shn-binding sites using DNaseI footprinting assays. In these experiments we used the ability of the bacterially produced protein to bind an MBP1 oligo that resembles the optimal Shn sites, as a control for activity (Fig. 4A). As illustrated in Fig. 4B, Shn DBD2 protects two regions within the *Ubx* B fragment. The S1 region that extends from nucleotides 458 to 487 shows a slightly higher affinity for Shn, compared to the second binding site (S2) that spans nucleotides 524 to 538 (numbering according to Saari and Bienz, 1987). Both protected areas contain sites with a 6/7 match to the GGGG and CCC motifs in the consensus Shn binding sites (Fig. 4C, see Fig. 3). The central portion of the sites showed a poor match to the consensus, indicating that these nucleotides may be less critical for binding. Shn DBD1 also showed protection of the same regions in *Ubx* B, consistent with our finding in site selection experiments that both sets of fingers recognize closely related sequences (data not shown).

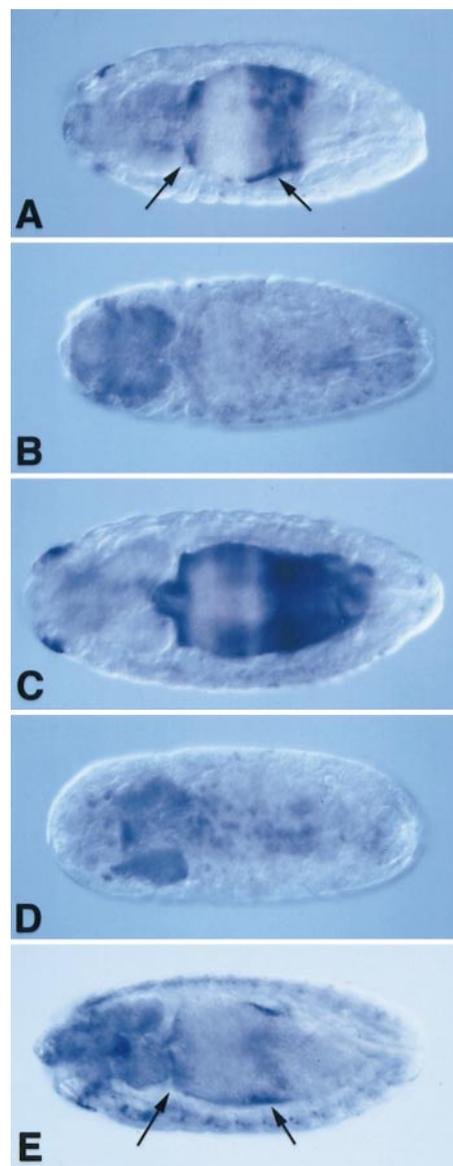


**FIG. 4.** DNaseI footprint analysis with Shn. Bacterially expressed GST-fusion protein containing the second pair of Shn finger domains binds the human MBP-1 consensus binding site (A), and the *Drosophila* Ubx B enhancer fragment (B). Sequencing ladders are indicated. The following lanes are with no protein added (0), or increasing amounts of protein in microliters. The regions from 458 to 487 and 524 to 538 in the noncoding strand of Ubx B are protected by Shn, and are bracketed at right as S1 and S2 (numbering according to Saari and Bienz, 1987). (C) Sequence alignment of Shn-binding sites in Ubx B. The numbers denote the extent of the protected region while the nucleotides correspond to the core sequence resembling the consensus. Black boxes represent identities with the optimal binding sites (see Fig. 3).

### *Shn* Activity Is Required for Induction of *Ubx* in the Midgut

*dpp* expression in parasegment 3 (ps3) and ps6–7 of the visceral mesoderm is required for induction of *Ubx* expression, and as mentioned earlier, transcription of the endogenous gene is lost in *shn* mutants (Grieder et al., 1995). While the number of visceral mesodermal cells is reduced in embryos lacking *shn* activity, this is unlikely to be the cause for loss of *Ubx* expression, since other genes such as *Sex combs reduced* that are expressed in the visceral mesoderm can be detected in *shn*<sup>-</sup> embryos (J. Torres-Vazquez and K. Arora, unpublished observations). We wished to assess whether the Ubx B reporter also required *shn* function for *dpp*-responsive expression. In wild-type embryos Ubx B-LacZ is expressed in a narrow anterior stripe in the visceral mesoderm in ps3 and in a broader posterior stripe encompassing ps6–9 (Fig. 5A; Thuringer et al., 1993). The broader posterior stripe represents a response to both Dpp signaling in ps6–7 and Wg signaling in ps8–9

(Thuringer et al., 1993). We found that in embryos lacking *shn* protein (*shn*<sup>P4738</sup>), Ubx B expression was lost at all sites, suggesting that Shn is required to mediate the transcriptional response to Dpp (Fig. 5B). However, it has been



**FIG. 5.** Expression of Ubx B in wild-type and *shn* mutant embryos. Dorsal view of 12- to 15-h-old embryos, stained with anti-LacZ antibody to visualize expression driven by wild-type (A–D) or a mutant Ubx B enhancer (E). Embryos in B and D are homozygous *shn*<sup>P4738</sup> mutants, the remainder are wild-type. Anterior is to the left. Ubx B drives expression in the visceral mesoderm in a narrow anterior domain in ps3 and a broader posterior domain spanning ps6–9, marked with arrows in (A). The reporter is expressed ectopically in response to heat shock Dpp (C). *shn* mutant embryos show a complete loss of reporter gene expression (B), that is not recovered even in response to ectopic Dpp (D). Mutations in Shn-binding sites in the Ubx B reporter (BS1S2) result in reduced staining (see arrows in E).

established that *dpp* expression is maintained through an indirect autoregulatory loop that involves positive feedback from Ubx expression in the same cells. In addition, *dpp* expression in ps7 is required to maintain *wg* expression in the adjacent cells of ps8 (reviewed in Bienz, 1997). Mutations in *shn* disrupt these autoregulatory interactions and result in loss of *dpp* expression in the midgut (Arora *et al.*, 1995; Grieder *et al.*, 1995). The absence of reporter gene expression in ps8–9 in *shn* mutants may also be attributed to the loss of *dpp* expression and its effect on maintenance of *wg* transcription. These observations raise the possibility that loss of Ubx B-LacZ in *shn* mutants could be an indirect consequence of the loss of *dpp* transcription. To test this, we provided Dpp exogenously in *shn* mutant embryos using a heat shock promoter and assayed reporter gene expression. In a wild-type background ectopic Dpp results in induction of Ubx B in an expanded domain (Fig. 5C). However in *shn* mutants, we observed a complete lack of LacZ staining in the visceral mesoderm; i. e., reporter gene expression was not recovered even when Dpp was exogenously supplied (Fig. 5D). These results indicate that Shn acts downstream of Dpp in the visceral mesoderm and is obligatorily required for Ubx B transcription.

In order to examine the contribution of Shn binding to Ubx B expression, PCR-based mutagenesis was used to introduce base substitutions in the Shn sites S1 and S2 (see Materials and Methods). The Ubx BS1S2 fragment was tested by footprint analysis and does not show protection by Shn over the range of concentrations that bind wild-type Ubx B (data not shown). Transgenic flies carrying a reporter mutant for both sites were generated by germline transformation and analyzed. As seen in Fig. 5E, mutation of the Shn sites significantly reduced the domain of expression in ps3 and lowered the level of transgene expression in ps 6–9. Similar results were obtained with a construct that contained mutant S1 sites but was deleted for S2 (data not shown). Transcription of the reporter in the gastric caeca (ps3) appears more sensitive to the loss of Shn sites, suggesting that these cells may require higher levels of Dpp signaling. This is consistent with the loss of Ubx expression in ps3 but not in ps7 in weak *dpp* alleles such as *dpp<sup>sd</sup>*, as well as in embryos lacking zygotic *Mad* (Immergluck *et al.*, 1990; Panganiban *et al.*, 1990a, b; Waltzer and Bienz, 1999). Taken together our results provide *in vivo* evidence that the Shn-binding sites in Ubx B are important for Dpp responsiveness.

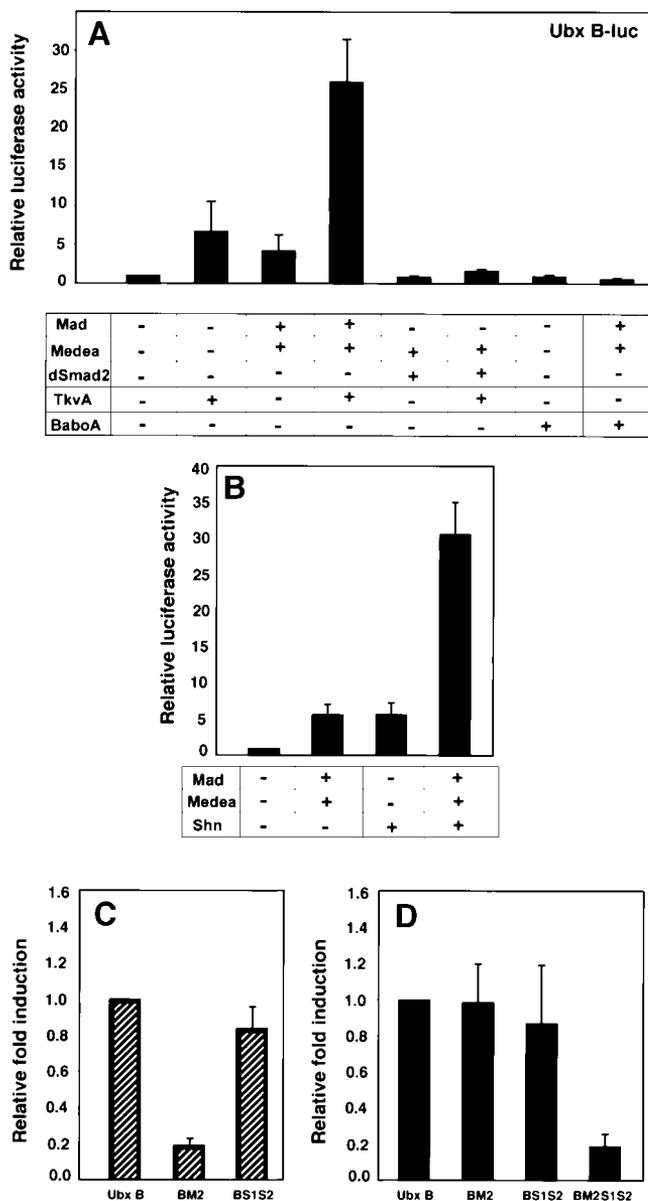
### **Shn and Mad/Medea Show Synergistic Transcriptional Activation**

Although mutations in the binding sites for Mad result in more severe loss of Ubx B expression compared to that caused by mutations in the Shn sites, in neither case is the expression abolished, raising the possibility that inputs from both proteins contribute to the regulation of Ubx B. There is increasing evidence that protein-protein interactions between Smads and accessory transcriptional factors can result in cooperative binding and synergistic transcrip-

tion of reporter genes (reviewed in Whitman, 1998; Zhang and Derynck, 1999). The fact that a Ubx B reporter that lacks Shn-binding sites (BS1S2) shows residual staining, while Ubx B expression is completely absent in *shn* mutant embryos (compare Figs. 5B, D, with E), suggests that loss of Shn protein has more severe consequences than loss of Shn-binding sites. In order to determine whether protein-protein interactions as well as DNA-binding contribute to activation of Ubx B by Shn and Mad, we developed an assay to study the nuclear response to Dpp signaling.

We cloned the B enhancer upstream of a minimal promoter driving expression of the luciferase gene (Ubx B-Luc) and examined its activity in cultured cells (see Materials and Methods). This reporter shows very low levels of basal expression in the BMP-responsive C3H10T1/2 cells (Hata *et al.*, 2000). As shown in Fig. 6A, cotransfection with Mad/Medea resulted in only a slight elevation of luciferase activity. However coexpression of Mad/Medea with constitutively activated TkvA resulted in a dramatic 25-fold increase in promoter activity relative to the basal response. In other words, coexpression of all three components caused a 5-fold stronger stimulation than expression of either Mad/Medea or TkvA alone. The response to TkvA was dependent on Mad and Medea since transfection with the receptor alone lead to only a small increase in transcription over basal levels, perhaps due to phosphorylation of endogenous BMP-specific Smads (Fig. 6A). Stimulation of Ubx B-Luc is Dpp/BMP pathway-specific since substitution of Mad with dSmad2, a *Drosophila* Smad that is involved in activin signaling (Brummel *et al.*, 1999), was not effective. Likewise cotransfection of a constitutively active form of the activin type I receptor Baboon (Babo), with Mad/Medea had little effect on reporter gene activity (Fig. 6A).

Previous studies have shown that several TGF- $\beta$  and activin response elements can be stimulated in a ligand-independent manner by the overexpression of specific Smads and their coactivators (reviewed in Zhang and Derynck, 1999). We therefore examined whether coexpression of Shn with Mad and Medea could enhance transcriptional activation of Ubx B-Luc. Under the conditions of our assay expression of Shn or Mad/Medea alone elicited a weak transcriptional response. However coexpression of all three proteins resulted in a 32-fold induction of reporter gene activity relative to the basal response (Fig. 6B). This is a 6-fold increase over the response to either Shn or Mad/Medea alone. More strikingly this induction is 3-fold greater than the expected additive response to expression of the individual proteins. To test the importance of Mad and Shn DNA-binding to synergistic activation, we initially generated a luciferase reporter construct lacking both Mad sites known to be required for expression in the embryo (Ubx BM2; see Szuts *et al.*, 1998). As anticipated, we found that the response of Ubx BM2-Luc to stimulation by TkvA and Mad was significantly reduced when compared to wild-type Ubx B (Fig. 6C). Interestingly, however, deletion of the Mad-binding sites in BM2 did not affect the induction of reporter activity by Mad/Medea in the presence of Shn



**FIG. 6.** Transcriptional activation of Ubx B-luciferase in mammalian cells by Shn and Mad. (A) The Ubx B-luc reporter is activated in a Dpp pathway-specific manner. C3H10T1/2 cells were transfected with the reporter either alone or in combination with expression vectors as indicated. Luciferase activity in cell lysates was measured 2 days posttransfection. Ubx B-luc is activated 25-fold by coexpression of TkvA and Mad/Medea, but not by components in the activin pathway. (B) Synergistic effect of overexpression of Shn and Mad/Medea. Neither Shn nor a combination of Mad/Medea stimulated significant transcription of Ubx B-luc, but coexpression resulted in a 32-fold induction of reporter activity. (C, D) Response of wild-type and mutant Ubx B-luc reporter constructs. BM2 has mutant Mad-binding sites, BS1S2 lacks Shn sites, and BM2S1S2 lacks sites for both factors. Experiments in (C) represent stimulation in response to TkvA and Mad, while experiments in (D) are responses to overexpression of Shn and Mad/Medea. The activities of the mutant reporters are expressed relative to wild-type Ubx B-luc. Data are the average  $\pm$  SEM of more than five independent experiments carried out in duplicate.

(Fig. 6D). In analogous experiments using a Ubx BS1S2-Luc reporter, loss of the Shn-binding sites only marginally affected the cooperative response to Shn and Mad/Medea (Fig. 6D). These results could indicate that synergistic transcriptional activation by overexpression of Shn and Mad/Medea does not depend entirely on their ability to bind DNA, but involves cooperative protein-protein interactions. To test this, we constructed a reporter that lacked both Mad as well as Shn-binding sites (Ubx BM2S1S2-luc). As seen in Fig. 6D, the response of Ubx B to overexpression of Mad/Medea and Shn is strongly reduced in the double mutant. We conclude that binding sites for either Mad or Shn are sufficient to mediate synergistic activation of the Ubx B reporter. However, when neither protein can bind the enhancer, it is no longer possible to elicit a transcriptional response. While the data in Fig. 6D may be interpreted as redundancy for Mad/Medea and Shn in stimulating UbxB transcription, this view is contradicted by the fact that expression of either protein alone clearly does not stimulate maximal response of the UbxB reporter (Fig. 6B). Taken together our data indicate that Shn can act as a transcriptional coactivator with Mad to regulate the expression of the Ubx B enhancer.

## DISCUSSION

The transcriptional response to signaling by TGF- $\beta$ -related ligands is dependent on three distinct properties of Smad proteins, their capacity to function as transactivators, their ability to bind DNA in a sequence-specific manner, and their interaction with other factors that modulate activity. The carboxy-terminal region of Smads contains a transactivation domain that stimulates gene expression when fused to a heterologous DNA-binding domain (reviewed in Massagué, 1998; Whitman, 1998). Smads have also been shown to bind short motifs related to the CAGAC sequence with weak affinity (Dennler *et al.*, 1998; Kim *et al.*, 1997; Zawel *et al.*, 1998). The low complexity of the target sequence and the relatively weak affinity for DNA displayed by Smad proteins indicates that they may require additional spatially and temporally regulated factors in order to increase the specificity and/or the range of the transcriptional response. Consistent with this, several transcription factors that interact with Smads and promote the transcription of TGF- $\beta$  and activin responsive genes have been identified (reviewed in Whitman, 1998). In contrast, the only transcription factor known to complex with BMP-specific Smads and mediate transcriptional activation is the vertebrate zinc finger protein OAZ (Hata *et al.*, 2000). In this study we provide evidence that Shn acts as a cofactor for Mad and plays an analogous role in mediating the response to the BMP ligand Dpp. This is the first evidence that BMP signaling in flies requires a direct interaction of Mad with a partner transcription factor.

We have demonstrated that Shn and Mad associate directly using several independent assays. Similar results have been reported in a recent study (Udagawa *et al.*, 2000).

The ability of the two proteins to interact does not appear to require signaling-dependent modification of either partner, since Shn and Mad associate both in yeast and *in vitro*. In addition in GST pull-down experiments, Shn can bind a truncated form of Mad lacking the carboxy-terminal serine residues that are targets for receptor phosphorylation, suggesting that Mad activation is not critical for interaction with Shn (C. Hogan and R. Warrior, unpublished data). While Shn and Mad clearly have an inherent affinity for each other, it is likely that their interaction is signaling dependent *in vivo*. In the embryo Shn is confined to the nucleus, while Mad is primarily cytoplasmic in the absence of Dpp signaling (Fig. 1B; Newfeld *et al.*, 1996; Staehling-Hampton *et al.*, 1995; K. Arora, unpublished data). Since Mad only enters the nucleus in response to Dpp signaling, the interaction between the two proteins *in vivo* is likely to be regulated by their localization to separate subcellular compartments. Consistent with this, coimmunoprecipitation of Shn with Mad is more effective under conditions that mimic Dpp signaling, such as in the presence of TkvA that causes nuclear translocation of higher levels of Mad (Fig. 1C; Hoodless *et al.*, 1996). Since it has been shown that in COS cells Mad is partially localized to the nucleus, an interaction detected between Shn and Mad under these conditions is not unexpected (Hoodless *et al.*, 1996; Wisotzkey *et al.*, 1998). In addition it is possible that in this assay Shn could drag Mad into the nucleus by virtue of their interaction, as has been shown for the proteins  $\beta$ -catenin and LEF-1 in the Wnt pathway (Behrens *et al.*, 1996). However, it is important to note that such a mechanism cannot be the basis for the synergistic response to Shn and Mad observed in 10T1/2 cells, since it does not explain the responsiveness of the BM2 reporter (that lacks Mad-binding sites) when both proteins are coexpressed.

We have identified three discrete regions in Shn that interact with the carboxy-terminal MH2 domain of Mad (Fig. 2). Sequence comparison failed to reveal any homology or structural similarity with Smad interaction domains that have been characterized in other proteins. It is conceivable that each MID functions independently, allowing a single Shn molecule to associate with more than one molecule of Mad. This is an attractive possibility since it has been postulated that signaling triggers the formation of a hexameric complex consisting of a Smad4 trimer and a receptor-regulated Smad trimer (Shi *et al.*, 1997). Alternatively, the three domains in Shn could combine to form a single high-affinity binding site for Mad. Interestingly the Shn polypeptides that interact with Mad can also mediate homomeric interactions, raising the possibility that Shn dimerization could compete with the association between Shn and Mad *in vivo*.

Shn contains three C<sub>2</sub>H<sub>2</sub> zinc finger domains. We find that the first and the second paired finger domains display overlapping DNA-binding specificity, although they share only 53% amino acid identity (Fig. 3). Both optimal binding targets resemble the NF $\kappa$ B consensus site 5'-GGGRNNYYCC-3' and closely correspond to the sequences recognized by MBP-1 and

MBP-2 (Baldwin *et al.*, 1990; Fan and Maniatis, 1990; Rustgi *et al.*, 1990; van't Veer *et al.*, 1992). Zinc finger proteins typically utilize multiple fingers to bind DNA, and it is believed that each individual finger contacts the major groove through an  $\alpha$ -helical region. Residues at four specific locations in the  $\alpha$ -helix play a critical role in base recognition (Elrod-Erickson and Pabo, 1999; Wolfe *et al.*, 1999). Significantly, we find that 7 of the 8 residues at the predicted contact sites are identical within the first and second pair of finger domains, thus providing an explanation for their overlapping binding specificity. A recently identified Smad-interacting protein (SIP1), implicated in the regulation of the activin-responsive Xbra promoter, also contains multiple dispersed zinc finger DNA-binding domains (Verschuere *et al.*, 1999). As in the case of Shn, both zinc finger domains in SIP1 have similar DNA-binding specificities, and a single molecule of SIP1 can bind two sites 24–44 bp apart, with each finger domain recognizing a single site (Remacle *et al.*, 1999). The presence of two protected regions 66 bp apart in the Ubx B fragment (see Fig. 4B) raises the possibility that Shn shares this mode of binding, and that each set of fingers binds adjacent regions in the target promoter. This could result in cooperative interactions that increase the affinity of the full-length Shn protein for enhancer elements *in vivo*. The carboxy-terminal zinc finger triad is unique to Shn and is not represented in its mammalian homologs. We have not been able to detect DNA binding by these fingers in site selection assays, raising the possibility that this domain does not contribute to Shn DNA-binding specificity (H. Dai and R. Warrior, unpublished data). Zinc finger domains have also been implicated in RNA and protein-protein interactions and it is possible that the carboxy-terminal finger triad has a non-DNA-binding function (reviewed in Mackay and Crossley, 1998).

Phenotypic analyses have implicated Shn in *dpp* signaling during midgut morphogenesis and shown that expression of several *dpp* target genes, including *Ubx*, *labial*, *wg*, and *dpp* itself, are dependent on Shn activity (Arora *et al.*, 1995; Grieder *et al.*, 1995; Staehling-Hampton *et al.*, 1995). Transcription of the Ubx B reporter in ps3 as well as in ps6–9 is completely lost in *shn* mutants. Furthermore, expression cannot be restored in midgut cells lacking Shn even when excess Dpp is supplied exogenously, reinforcing the idea that *shn* is obligately required for the transcriptional response to Dpp at both these sites (see Fig. 5). We have identified Shn-binding sites in the Ubx B enhancer element supporting the idea that Shn plays a direct role in the transcriptional response to Dpp. Elimination of Shn-binding sites resulted in a significant reduction in reporter gene expression both in transient transfection assays and in *Drosophila* embryos, indicating that these sites contribute to mediating the response to Dpp.

The fact that Ubx BS1S2 lacking Shn-binding sites retains some expression, while there is no expression of wild-type Ubx B in *shn* mutants, implies that Shn may influence the response to Dpp independent of its ability to bind the Ubx B enhancer. One possibility is that in addition to activating *Ubx*, Shn also regulates other proteins that affect transcrip-

tion of the reporter. Formally Shn could activate a positive regulator of Ubx B, or restrict the expression of a repressor of Dpp signaling. Candidate regulators that have been shown to provide positive inputs to the Ubx B enhancer include CREB and CBP (Waltzer and Bienz, 1999). In addition, recent studies have revealed an important role for negative transcriptional regulation in the expression of Dpp target genes. Both in embryos and in wing imaginal discs, Dpp signaling has been found to inhibit the expression of a nuclear protein Brinker (Brk) that represses *dpp* target genes. Shn could contribute to Ubx B activation by sterically hindering or inhibiting Brk binding to the promoter. Interestingly, Mad is required both for relieving Brk-mediated repression, as well as direct activation of a subset of *dpp* targets in the wing disc (Campbell and Tomlinson, 1999; Jazwinska et al., 1999a, b; Minami et al., 1999). Given that Mad and Shn can interact directly, it is also conceivable that they act together to repress Brk (Torres-Vazquez et al., 2000). Thus Shn/Mad could contribute to Ubx B expression by direct activation as well as through repression (or inhibition) of Brk.

An alternative basis for the stringent requirement for Shn protein versus Shn-binding sites is revealed by analysis of reporter gene expression in cotransfection assays. These results make a compelling argument in support of the idea that Shn and Mad act cooperatively to regulate Ubx B transcription. We find that in mammalian 10T1/2 cells the Ubx B enhancer responds effectively to conditions that mimic Dpp signaling, and that coexpression of Shn and Mad/Medea results in synergistic activation (Fig. 6B). Given that Shn and Mad interact directly, it is likely that they form a complex at the promoter that may be critical in activating transcription of target genes. Interestingly, the presence of binding sites for either Shn or Mad alone was sufficient to mediate a synergistic response when both proteins were overexpressed (Fig. 6D). This suggests that the Shn/Mad interaction is strong enough to mediate the formation of a functional complex that can be tethered to the promoter by either partner. Assembly of such a complex is likely to depend both on protein-protein interaction and on DNA binding. Loss of DNA binding by any one partner may not be critical when both proteins are present in excess. In the embryo the sensitivity of different Dpp target genes to Shn and Mad would depend on the arrangement and relative affinities of the binding sites for the two proteins, as well as their nuclear concentrations. This may explain why loss of binding sites for Shn (or Mad) has a greater impact on reporter gene expression in the embryo compared to cultured cells, where luciferase activity is not impacted unless sites for both Shn and Mad are abolished. A similar relationship has been described between Smad3/4 and the AP-1 family of proteins. Smad3 and 4 stimulate transcription of a 3TP-lux derived reporter that contains both AP-1 and Smad-binding sites. Mutations in the Smad sites do not affect the ability of Smads3/4 to activate this reporter, and it is thought that they mediate transcription in the absence of DNA binding by forming a complex with

Jun proteins at the AP-1 sites (Liberati et al., 1999; Zhang et al., 1998).

Several studies suggest that involvement of partner transcription factors may be a common motif in Smad-dependent gene expression. For example, FAST1 is essential in mediating activin and TGF- $\beta$ -responsive gene expression in *Xenopus* and in mammals and, like Shn, has both DNA-binding and Smad-interacting functions (Yeo et al., 1999). As described above, the Jun components of AP1 play an analogous role in some TGF- $\beta$ -dependent transcriptional responses (Liberati et al., 1999; Zhang et al., 1998). In this context, it may be significant that CRE-binding sites that can be recognized by heterodimers of CREB with Jun or Fos are adjacent to and overlap with Mad sites in the Ubx B element (Eresh et al., 1997; Szuts et al., 1998). A second class of Smad-associated transcription factors affects signaling without direct protein-protein interactions. An example of this category of Smad partners is TFE3, a mammalian helix-loop-helix protein that acts synergistically with Smad3/4 to enhance the activity of a TGF- $\beta$ -responsive reporter (Hua et al., 1998). In *Drosophila* the homeobox protein Tin may have a similar function since it binds its own promoter and is required for Mad-dependent expression of *tin* (Xu et al., 1998). Functional interactions have also been demonstrated between Smads and non-DNA-binding transcriptional coactivators such as CREB-binding protein (CBP) and MSG (Janknecht et al., 1998; Pouponnot et al., 1998; Shen et al., 1998; Shioda et al., 1998; Topper et al., 1998; Waltzer and Bienz, 1999). The ability of Smads to interact with transcriptional repressors such as SIP1, Evi-1, and Gli3 suggests that the choice of partner proteins can result in negative as well as positive effects on gene expression (Kurokawa et al., 1998; Remacle et al., 1999).

While several Smad-interacting transcription factors have been identified in the TGF- $\beta$  and activin pathways, Shn is the first example of a DNA-binding Smad cofactor involved in Dpp signaling. Although mutations in *shn* have a weaker phenotype than *dpp* null embryos, *shn* is required for several Dpp/Mad-dependent processes including specification of the mesoderm, dorsal closure, and tracheal development. Recent experiments indicate that *shn* activity is also essential for adult patterning and expression of *dpp* target genes such as *optomotor blind*, *spalt*, and *vg* in wing imaginal discs (J. Torres-Vasquez and K. Arora, unpublished data). Consistent with this we find that the Dpp-responsive *vg* quadrant enhancer can be synergistically stimulated by coexpression of Shn and Mad in our reporter assay. The presence of Shn- and Mad-binding sites in the *vg* promoter suggests that cooperative interactions between these proteins could be involved in the transcription of other *dpp* target genes (H. Dai and R. Warrior, unpublished data). Collectively, these data imply that Shn may act primarily by increasing the sensitivity rather than the specificity of the transcriptional response. However, the DNA-binding ability of Shn could increase selectivity at the level of promoter recognition, in that only the subset of genes that

contain both Shn- and Mad-binding sites would be candidates for regulation. Finally, it is possible that the MBP proteins that are structurally related to Shn and have similar DNA-binding specificity play analogous roles in BMP signaling in vertebrates.

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