Schnurri is required for Drosophila Dpp signaling and encodes a zinc finger protein similar to the mammalian transcription factor PRDII-BF1

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Abstract

Cytokines of the TGF beta superfamily regulate many aspects of cellular function by activating receptor complexes consisting of two distantly related serine/threonine kinases. Previous studies have indicated that Drosophila dpp uses similar signaling complexes and strictly requires the punt and thick veins receptors to transduce the signal across the membrane. Here, we show that the schnurri (shn) gene is required for many aspects of dpp signaling. Genetic epistasis experiments indicate that shn functions downstream of the dpp signal and its receptors. The shn gene encodes a large protein similar to a family of mammalian zinc finger transcription factors. The shn protein might therefore act as a nuclear target in the dpp signaling pathway directly regulating the expression of dpp-responsive genes.
schnurri Is Required for Drosophila Dpp Signaling and Encodes a Zinc Finger Protein Similar to the Mammalian Transcription Factor PRDII-BF1

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Summary

Cytokines of the TGFβ superfamily regulate many aspects of cellular function by activating receptor complexes consisting of two distantly related serine/threonine kinases. Previous studies have indicated that Drosophila dpp uses similar signaling complexes and strictly requires the punt and thick veins receptors to transduce the signal across the membrane. Here, we show that the schnurri (shn) gene is required for many aspects of dpp signaling. Genetic epistasis experiments indicate that shn functions downstream of the dpp signal and its receptors. The shn gene encodes a large protein similar to a family of mammalian zinc finger transcription factors. The shn protein might therefore act as a nuclear target in the dpp signaling pathway directly regulating the expression of dpp-responsive genes.

Introduction

Cell-to-cell signaling plays a central role in the coordinated development of cells and tissues in multicellular organisms. Various families of secreted signaling molecules have been characterized that transmit information from one cell to another. The transforming growth factor β (TGFβ) superfamily represents a particularly important class of signaling molecules (Kingsley, 1994). Members of this superfamily have been found in many species from Drosophila melanogaster to Homo sapiens, and their functions range from the control of cell division to the determination of distinct cell fates (Roberts and Sporn, 1993; Wall and Hogan, 1994).

Transmembrane receptors have been identified that are able to bind TGFβ ligands and appear to transduce their signal across cell membranes (Massague, 1992; Kingsley, 1994). In the case of TGFβ itself, biochemical studies have shown that its receptor complex is composed of two distantly related transmembrane serine/threonine kinases called type I and type II receptor (Massague et al., 1994). TGFβ binds directly to the type II receptor, which is a constitutively active kinase. This binding to the type II receptor leads to its association with the type I receptor, resulting in a transphosphorylation of the type I receptor at a conserved, type I-specific GS domain (Wranät al., 1994). This allows the propagation of the signal to putative downstream components. These downstream components are as yet unidentified, and the direct cytoplasmic and nuclear consequences of TGFβ receptor complex activation remain obscure.

The Drosophila decapentaplegic (dpp) gene encodes a member of the TGFβ superfamily of signaling molecules (Padgett et al., 1987) most closely related to the vertebrate bone morphogenetic protein 2 (BMP2) and BMP4 proteins. Molecular and genetic analysis has led to the identification of several transmembrane serine/threonine kinases that appear to act as dpp receptors in vivo. thick veins (tkv) and saxophone (sax) encode type I receptors, whereas punt encodes a type II receptor (Xie et al., 1994; Nellen et al., 1994; Brummel et al., 1994; Penton et al., 1994; Ruberte et al., 1995; Letsou et al., 1995). It has been shown that the tkv and punt receptors are equally indispensable in transducing the dpp signal across the membrane (Ruberte et al., 1995) and therefore presumably act in concert to transmit the signal to unidentified downstream components. Strikingly, neither tkv nor punt function can be replaced by other extant type I or II receptors (Ruberte et al., 1995).

The use of Drosophila genetics should allow the identification of mutations in genes that are essential to implement the molecular and cellular consequences of dpp receptor stimulation. Such newly identified components are likely to have counterparts in higher vertebrates, as indicated by the conservation of similar receptor complexes in Drosophila and vertebrates. The isolation of genes required for the dpp pathway may therefore identify general components of the TGFβ and BMP signaling pathways.

We report here the characterization of the gene schnurri (shn; Nüsslein-Volhard et al., 1984) and show that its product is required for many aspects of dpp signaling during Drosophila development. The phenotypes caused by mutations in shn are strikingly similar to those seen in embryos that lack zygotic punt or tkv activities. shn mutants are unable to respond to ectopic dpp expression in the embryonic midgut, suggesting that shn is an essential component required to implement dpp signaling in the endoderm and the visceral mesoderm. We show that shn encodes a large putative transcription factor that contains seven zinc fingers and is similar to a family of previously characterized vertebrate proteins. Therefore, shn might represent a direct target of the dpp signaling pathway and respond to its stimulation by the activation or repression of dpp-responsive genes.

Results

To identify genetic components involved in the transmission and interpretation of signals elicited by the TGFβ homolog dpp, we have analyzed genes required for dorsal closure (Wieschaus et al., 1984; Nüsslein-Volhard et al.,
1984; Jürgens et al., 1984), a process blocked by mutations in the dpp type I receptor gene tkv and the dpp type II receptor gene punt. Among the 20 complementation groups displaying a so-called dorsal open phenotype, we were looking for those that showed additional tkv/punt/dpp-like defects (see below). shn mutant embryos showed a number of such defects. This prompted us to study in detail the genetic requirements of shn during embryonic development and to compare its genetic functions with those of dpp and its receptors.

Similar Midgut Defects in schnurri and dpp Mutants

dpp, in conjunction with tkv and punt, is required for midgut morphogenesis and controls the expression of several genes in the visceral mesoderm and the underlying endoderm (Bienz, 1994; Crabtree et al., 1992). Genes whose expression has been shown to depend on dpp activity include the homeotic genes Ultrabithorax (Ubx) and labial (lab), the wingless (wg) gene, and the dpp gene itself (Immerglick et al., 1990; Panganiban et al., 1990; Reuter et al., 1990; Hursh et al., 1993). Expression of all of these dpp target genes is altered in shn mutants: Ubx expression is virtually undetectable (Figure 1B), and dpp expression (Figure 1D) and wg expression (see Figure 3D) are absent in the visceral mesoderm; shn embryos also fail to express lab in the adjacent endodermal cells (Figure 1F). In addition, homozygous shn embryos lack the second midgut constriction and do not form gastric ceca (data not shown). All these phenotypes are observed in dpp~4 mutants that lack dpp expression in the developing midgut (Immerglick et al., 1990; Panganiban et al., 1990), and in mutants with a zygotic loss of dpp receptor activities (Nellen et al., 1994; Penton et al., 1994; Affolter et al., 1994; Ruberte et al., 1995). Thus, shn mutants mimic dpp~4, tkv, and punt mutant phenotypes in the developing midgut, raising the possibility that shn is required for dpp signaling in this tissue.

schnurri Is Essential for Mediating the Response to Dpp in the Endoderm

The lack of lab expression observed in shn mutants (Figure 1F) could indicate that shn is essential for mediating lab induction in endodermal cells in response to the dpp signal presented by cells of the visceral mesoderm. However, our observation that dpp is not expressed in the visceral mesoderm of shn embryos (Figure 1F) could suggest that the absence of lab expression is an indirect consequence of the lack of dpp in the adjacent cell layer. To distinguish between these two possibilities, we restored dpp expression in the developing midgut of shn mutants and analyzed the induction of lab transcription in the endoderm.

Transcription of the homeotic gene lab is normally limited to the central portion of the midgut endoderm, which underlies the dpp-secreting portion of the visceral mesoderm (Immerglick et al., 1990; Panganiban et al., 1990). In wild-type embryos, ubiquitous expression of dpp under the indirect control of a heat shock promoter leads to an expansion of the lab domain in the midgut such that virtually all endodermal cells accumulate high levels of lab protein (Figure 2A; Ruberte et al., 1995; see also Staehling-Hampton and Hoffmann, 1994). In contrast, heat-induced ubiquitous expression of dpp in shn mutant embryos did not result in the activation of the lab gene, neither in its normal domain in the central midgut nor elsewhere in the midgut (Figure 2B). This demonstrates that dpp, even when present at high levels throughout the embryo, is unable to induce expression of the lab gene in the absence of the shn gene product.

lab induction in the embryonic midgut is the result of cell-cell interactions between two germ layers, the visceral mesoderm and the endoderm. It has been proposed that the dpp signal directly acts to transfer the inducing effect from the visceral mesoderm to the adhering endoderm (Immerglick et al., 1990; Panganiban et al., 1990). Alternatively, it is possible that a different secreted protein, whose production in mesodermal cells relies on Ubx and dpp, constitutes the visceral mesoderm-to-endoderm signal. To exclude the alternative possibility, we have tested whether dpp can induce lab expression in endodermal cells in the absence of mesodermal structures.

As shown in Figure 2, ectopic dpp expression leads to endodermal lab accumulation in tinman mutants (Figure 2C), which lack the visceral mesoderm, and in twist mu-
tants (Figure 2D), which lack all mesodermally derived structures. Thus, it appears that the effects of ectopic dpp on lab expression are a direct consequence of the interaction of the dpp signaling molecule with endodermal cells and are not mediated via other signals induced by dpp in the visceral mesoderm. We conclude that the failure of dpp to induce lab expression in shn mutants is due to a defect in endodermal cells. This demonstrates that shn is an essential component in the response of endodermal cells to the dpp signal.

**shnurri Also Mediates the Response to Dpp in the Visceral Mesoderm**

Previous studies have shown that ectopic dpp expression during midgut development induces the expression of Ubx and wg in the posterior visceral mesoderm (Figures 3A and 3C; Staehling-Hampton and Hoffmann, 1994). To find out whether such a dpp response occurs in the absence of shn activity, we analyzed the expression pattern of Ubx and wg in shn mutants after ubiquitous dpp expression. Even under these conditions, Ubx expression is undetectable in the visceral mesoderm of most homozygous shn− embryos; in the rare shn− embryos in which low levels of Ubx are discernable, the Ubx expression domain remains limited to the central midgut and is not expanded posteriorly (compare Figures 3A and 3B). Furthermore, unlike in sibling shn+ embryos, wg expression remains undetectable in the visceral mesoderm of homozygous shn− mutants after ectopic expression of dpp (Figure 3D); wg expression is thus neither induced nor expanded by such a treatment. These results indicate that cells of the visceral mesoderm require shn to respond to dpp.

**Maternal Function of schnurri**

The earliest function of dpp during embryogenesis is to specify distinct cell fates in the dorsal half of the early embryo in a dosage-dependent manner (Ferguson and Anderson, 1992; Wharton et al., 1993). To carry out this function, dpp relies on maternally and zygotically provided receptors, including punt, tkv, and sax (Ruberte et al., 1995; Nellen et al., 1994; Letsou et al., 1995).

To analyze whether zygotic shn activity is required for early dorsoventral patterning, we have examined the cuticular phenotype of shn mutant larvae. With the exception of the dorsal hypoderm (see below), most cuticle structures derived from dorsal regions of the blastoderm fate map...
are present in shn mutants (data not shown). In addition, the expression pattern of Krüppel, a marker for the determination and differentiation of the dorsal-most pattern element, the amnioserosa, is not affected in embryos that lack zygotic shn activity. Consistent with the correct specification of the amnioserosa, dpp expression in shn mutant embryos is indistinguishable from that observed in wild-type embryos: expression is restricted to the dorsal epidermis during germband extension and later excluded from cells that form the amnioserosa (data not shown). Thus, early dorsoventral patterning does not require zygotic shn activity.

Because shn transcripts are present in early syncytial embryos (see below), we analyzed the maternal requirement of shn for the specification of the dorsoventral axis (see Experimental Procedures). Cuticles from larvae lacking both maternal and zygotic shn gene function are more severely affected than those obtained from larvae that lack only zygotic gene activity; such cuticles completely lack the sclerotinized head skeleton at the anterior and the filzkörper at the posterior end. In addition, no residual dorsal hairs develop in the apparent absence of maternal and zygotic shn activity. At present, we do not know whether the rare shn-embryos laid from females carrying shn-germline clones also show defects in the very early specification of the dorsoventral axis, e.g., the determination of the amnioserosa. Further analysis using molecular markers will be required to study in more detail the maternal function of the shn gene.

**schnurri Is Required for Cell Differentiation in the Dorsal Ectoderm**

Later events leading to the determination and differentiation of dorsal ectodermal cells are greatly affected in zygotic shn mutant embryos. The pannier (pnr) gene is required for dorsal closure and is specifically expressed in the dorsal ectoderm (Figure 4A; Ramain et al., 1993; Winning et al., 1993). pnr expression, although normal during early stages of gastrulation, is absent from the dorsal-most cells after the beginning of germband retraction in shn-embryos (Figure 4B). At the same developmental stage, the dorsal and lateral stripes of dpp expression are nearly undetectable in shn mutants (Figure 4D). Even more strikingly, several cell types normally specified in the region of the dorsal ectoderm between the dorsal and lateral dpp stripe, e.g., tracheas and oenocytes, are absent in shn-embryos (compare Figures 4F and 4H with Figures 4E and 4G, respectively). wg expression, repressed in the dorsal epidermis during germband retraction in wild-type embryos (van den Heuvel et al., 1989; Figure 4I), is circumferential in shn mutants (Figure 4J). This is presumably
a result of the failure to specify dorsal and dorsolateral ectodermal cells properly. These observations indicate that the dorsal and dorsolateral ectodermal cells do not differentiate in shn mutants, consistent with the observed loss of dorsal hypoderm reported by Nüsslein-Volhard et al. (1984).

The above interpretation is strengthened by the changes we observed in shn mutant embryos with respect to the expression pattern of the Distalless (Dll) gene. At the extended germband stage, Dll is expressed dorsally in the mandibular, maxillary, and labial segments, and as ventrolateral dots in the first three thoracic segments (Cohen, 1990; Figure 4K). Consistent with the lack of dorsal markers in shn mutants, Dll expression is no longer detectable in the mandibular, maxillary, and labial segments (Figure 4L). In contrast, Dll expression is expanded considerably in the thoracic segments of shn- embryos and in most cases reaches the dorsal-most cells.

Thus, zygotic shn gene activity is required for the proper differentiation of dorsal ectodermal cells. Prior to the appearance of the first defects, dpp is expressed throughout the dorsal ectodermal region, and it is likely that the lack of shn activity interferes with the proper interpretation of the dpp signal in these cells (see Discussion).

Expression of schnurri during Embryogenesis

To examine the pattern of shn expression during embryogenesis, whole-mount embryos were hybridized with digoxigenin-labeled shn cDNA probes (see below). High and uniformly distributed levels of shn RNA are detected in early syncytiot eggs. During cellularization, the pattern of transcript distribution is extremely dynamic. Transcripts first accumulate along the dorsal side of the embryo, covering the anterior and posterior poles in a pattern very similar to the one observed for dpp transcripts (Figure 5A). During the late phases of cellularization, shn transcripts start to fade away from the posterior end and appear along the ventral side in the invaginating mesoderm (Figures 5B and 5C). From fertilization to the beginning of gastrulation, the expression pattern of shn is very similar to the expression pattern of the dpp type I receptor gene tkv (Affolter et al., 1994). During germ band extension, transcripts are present throughout the mesoderm (D). At the extended germ band stage and during germ band retraction, shn RNA is seen in all anterior and posterior endodermal midgut cells. Expression appears to be rather ubiquitous in these and later stages.

The schnurri Gene Encodes a Large, Putative Transcription Factor with Seven Zinc Fingers

To clone the shn locus, we made use of a shn allele (designated as shn+) we identified in the collection of P element–induced lethals generated by Karpen and Spradling (1992). We initially cloned genomic sequences flanking the single P element located at 47E. Subfragments of the rescued genomic DNA were then used to screen various cDNA and genomic libraries (for details, see Experimental Procedures).

The following lines of evidence argue that all the cDNAs we cloned (Figure 6A) derive from the shn locus. The P element insertion chromosome leads to embryonic lethality when heterozygous over all the available shn alleles. However, the phenotype and the lethality of shn+ mutants can be reverted to wild type by the mobilization of the single P element (data not shown). The P element appears to be inserted in an intron that separates the promoter from the main body of the coding sequences (see legend of Figure 6 and Experimental Procedures). The insertion of the P element at this site leads to the absence of detectable levels of transcripts in homozygous shn+ embryos (see Experimental Procedures). This indicates that the 15 kb P element sequences interfere with the proper expression of the transcription unit represented by the cDNAs we cloned.

The amino acid sequence derived from our shn cDNAs is shown in Figure 6B. shn encodes a conceptual protein of over 2500 amino acids. Computer-assisted similarity searches in the GenBank and EMBL databases revealed that shn shares two domains of high sequence similarity with a family of zinc finger transcription factors isolated from vertebrates (interferon-13-positive regulatory domain II–binding factor 1 [PRDL-BF1; Fan and Maniatis, 1990]; major histocompatibility complex–binding proteins 1 and 2 [MBP1, MBP2; van ‘t Veer et al., 1992]; uA-crystallin-binding protein 1 [uA-CRYBP1; Nakamura et al., 1990]; and human immunodeficiency virus enhancer–binding protein 1 [HIV-EP2; Nomura et al., 1991]). The regions of similarity include two sets of paired Cys2-His2 zinc fingers present in these proteins (see Figures 6B and 6C). In contrast with the transcription factors mentioned above, the shn protein has an additional set of paired zinc fingers at
Figure 6. Organization and Sequence of the shn Gene

(A) Molecular map of the cloned genomic and cDNA sequences flanking the P insertion. Above the solid horizontal line representing genomic DNA, the integration site of the P element in (2)04738 is indicated. All EcoRI (R), HindIII (H), and XbaI (X) sites to the right of the P insertion are shown. The two SphI (S) sites used to isolate the SphI probe are also shown. Below the genomic region, the position of the isolated cDNA is drawn. The direction of transcription in the shn locus is from left to right, as deduced from in situ hybridizations with strand-specific RNA probes. Five introns of less than 70 bp are not indicated on the genomic region. The integration site of the P element in Figure 6. Organization and Sequence of the shn Gene

(B) Schematic comparison of the derived amino acid sequences of shn and PRDII-BF1. The six Cys-His-zinc fingers are boxed and shaded. Amino acid residues identical to those in PRDII-BF1 are printed in bold letters. Apart from the two sets of paired zinc fingers, the predicted shn protein contains a region of 17 amino acids with 12 residues identical to those in PRDII-BF1. Approximately 300 amino acids C-terminal to this region, both proteins harbor an additional but less well-conserved zinc finger motif (Figures 6B and 6C).

Thus, the predicted shn gene product is a large protein zinc finger that shows high similarity to the human PRDII-BF1 protein, in both the overall alignment and the spacing of conserved protein motifs as well as in their amino acid composition. It has been demonstrated that each set of zinc fingers of PRDII-BF1 is able to bind DNA in a sequence-specific manner (Fan and Maniatis, 1990). The high degree of sequence identity between PRDII-BF1 and shn in these two conserved paired zinc fingers (73% in the first domain and 89% in the second) strongly suggests that the Drosophila shn protein binds DNA and acts as a transcriptional regulator.

Discussion

**schnurri** Phenotypes Resemble *dpp*, *tkv*, and *punt* Phenotypes

Previous studies have led to the identification of several receptors for the dpp signaling molecule, notably the indis-
The Zinc Finger Protein Schnurri Is Required for Dpp Signaling

797

pensable type I and type II receptors tkv and punt, respectively (Nellen et al., 1994; Brummel et al., 1994; Penton et al., 1994; Ruberte et al., 1995; Letsou et al., 1995). To search for additional components required for dpp signaling, we analyzed genes required for dorsal closure, a process that is blocked by mutations in either the tkv or the punt genes. C. Nüsslein-Volhard, E. Wieschaus, and colleagues (Wieschaus et al., 1984; Nüsslein-Volhard et al., 1984; Jürgens et al., 1984) isolated a number of zygotic lethal complementation groups that result in the failure to close the dorsal hypoderm. Only shn displayed additional phenotypes shared by mutations in dpp, tkv, and punt: the failure to activate expression of the dpp target genes Ubx, wg, and lab in the developing midgut. Although the lack of dpp itself in the visceral mesoderm of shn mutants could have been the primary defect leading to the absence of Ubx, wg, and lab expression, we have shown that this is not the case. By expressing dpp at high levels throughout the embryo, we showed that the cells of the endoderm are not able to respond to dpp in the absence of shn function. In this respect, shn mutants behave like embryos lacking the dpp receptors tkv and punt (Ruberte et al., 1995). Furthermore, loss of shn activity abolishes the ectopic dpp response in cells of another germ layer, the visceral mesoderm.

Mutations in shn lead to an additional striking phenotype: the failure to develop structures arising from the dorsal ectoderm (Figure 4). dpp is expressed in the entire dorsal ectoderm during germband extension (St Johnston and Gelbart, 1987; Jackson and Hoffmann, 1994). It is possible that the defects we observed in shn embryos are due to the failure of these cells to interpret the dpp signal. However, the lack of dpp mutations that specifically remove dpp expression in the dorsal ectoderm hinders a more direct analysis of this interpretation; its confirmation must await the isolation of such cis-regulatory dpp mutations.

Our analysis of shn mutant clones in the adult cuticle indicates that shn is also required for later dpp function. shn mutant cells in the wing, for example, fail to differentiate vein material (Zecca et al., 1995). Thus, the cell-autonomous requirement of shn for vein differentiation is consistent with a role downstream of the dpp vein-inducing function in late imaginal disc development.

The total absence of dpp gene activity causes a complete ventralization of the embryonic epidermis (Irish and Gelbart, 1987). Indistinguishable phenotypes result from the simultaneous loss of maternal and zygotic expression of punt and tkv (Nellen et al., 1994; Ruberte et al., 1995). Considering our findings that shn is strictly required for many aspects of dpp signaling, it is somewhat surprising that the loss of maternal and zygotic shn activity does not result in a dpp-null phenotype. The cuticle defects on the dorsal side of zygotically mutant larvae were enhanced after simultaneous removal of maternal and zygotic shn gene product. At present, we do not know whether this represents a defect in early dorsoventral patterning or whether it represents an enhanced (or total) failure to differentiate dorsal ectodermal cells properly in the absence of maternal gene product. Further studies are needed to determine the precise maternal function of the shn gene.

schnurri Encodes a Large Zinc Finger Transcription Factor

The identification of extended sequence similarities between shn and vertebrate transcription factors in two zinc finger DNA-binding domains strongly suggests that shn encodes a large transcription factor. The vertebrate proteins have been isolated on the basis of their ability to bind to cis-regulatory regions of various genes, including the human major histocompatibility complex class I gene, the rat α-1-antitrypsin gene, and the mouse αA-crystallin gene. However, the function of the vertebrate proteins in the regulation of these genes remains unknown, as the lack of genetic approaches has hindered thus far the isolation of direct target genes for these factors. It will be interesting to analyze whether these vertebrate proteins are also required for gene expression regulated by TGFβ superfamily members.

In Drosophila, response elements that can mediate dpp-dependent transcriptional regulation have been identified in the cis-regulatory control regions of the homeotic genes Ubx and lab (Thürringer et al., 1993; Thürringer and Bienz, 1993; Tremml and Bienz, 1992). Future studies will show

Figure 7. shn Activity Is Required for Vein Formation in the Developing Wing

(A) Wing containing multiple clones of shn mutant cells induced during mid-third instar larval period. The clones are simultaneously mutant for forked and therefore marked for analysis under high magnification. Mutant tissue fails to differentiate veins.

(B) Higher power magnification of a forked, shn mutant clone. The boundary of the clone is traced by a red line. The clone is situated on the dorsal wing surface. Magnification is three times higher than in (A).
whether the shn protein can interact directly with these regulatory elements and mediate dpp-regulated gene expression.

The Role of Schnurri in Dpp Signaling

Conceptually, shn might be required at various steps in dpp signaling. Shn could act as a transcription factor in the generation of the dynamic expression pattern of the dpp gene. Although dpp expression is altered in shn mutants at late stages (see Figure 4), we believe that this is either due to the failure of dpp to autoregulate its own transcription (via autocrine mechanisms requiring tkv and punt; see Hursh et al., 1993; Nellen et al., 1994; Ruberte et al., 1995) or due to secondary effects. High level, ectopic expression of dpp protein does not restore expression of dpp target genes in the visceral mesoderm and in the endoderm of shn· embryos (Figure 2). This indicates that genetically, shn acts downstream of dpp.

shn mutant phenotypes are also not caused by the failure to express the essential dpp receptors punt or tkv. We conclude this from the fact that the introduction of a chromosome containing functional rescue constructs for both of these two receptors does not ameliorate shn cuticle defects (see Experimental Procedures). Thus, shn does not act upstream of dpp or its receptors punt and tkv.

Our analysis of the epistatic relationship of shn with respect to dpp, tkv, and punt, in conjunction with our studies on the requirements of shn in the midgut and in the developing wing, indicates that shn acts in those cells that receive the dpp signal. shn might be an immediate-early gene, the transcription of which is activated as a result of the stimulation of the dpp receptor complex. However, the widespread expression of shn in the embryo argues strongly against this possibility. More likely, the shn protein might be a nuclear component of the dpp signaling cascade and act as a direct target of the signaling pathway. Such components have not been identified yet, and their isolation will greatly help to understand the molecular basis of cellular responses to members of the TGFβ superfamily. Future experiments, both at the genetic and the biochemical level, will reveal in more detail at which step in the interpretation of dpp signaling the shn gene product plays its indispensable role.

Experimental Procedures

Drosophila Strains

The P element line 12204738 (Karpfen and Spradling, 1992) did not complement the two available alleles of shn (Nüsslein-Volhard et al., 1984). All three alleles behave as embryonic lethals. Analysis of the developing tracheal system suggests that shn+ is a stronger allele than shn0, which in turn is stronger than 12204738 (data not shown). Cuticles of homozygous shn0 embryos are indistinguishable from those of shn0-DP(2R)ser(2), indicating that shn0 behaves as an apparent null allele. All defects were analyzed in shn0 embryos.

The construction and the use of the hs-GAL4 and the UAS-dpp transgenes is described by Ruberte et al. (1995). To analyze the effects of ectopic dpp expression in shn mutants, we constructed strains that contained a shn0 chromosome over a marked CyO balancer and that were homozygous for the hs-GAL4 or the UAS-dpp construct on the third chromosome. hs-GAL4- and UAS-dpp-containing strains were then crossed together. Embryos were collected, submitted to a 20 min heat shock treatment, and fixed 4 hr later for analysis.

To assay whether a chromosome containing both a hs-punt and a hs-tkv transgene could rescue shn cuticle phenotypes, a hs-punt, hs-tkv chromosome was introduced into a shn background and its rescue capacity tested by performing cuticle preparations and by assaying for embryonic lethality. The rescue chromosome could rescue neither shn cuticle defects nor the embryonic lethality caused by the shn mutation; however, the same rescue constructs fully rescued both of these phenotypes of punt or tkv mutations (see Ruberte et al., 1995).

Germline and Somatic Clones

To generate maternally and zygotically mutant shn embryos, we selected for homozygous mutant germline clones by using an autosomal [P{w+}] insertion (Chou et al., 1993; provided by N. Perrimon and T. B. Chou) on the FRT-42 chromosome and a recombinant FRT-42shn· chromosome. FLP-induced germline clone-bearing females were fertilized with shn·CyO males. Somatic clones were induced by using a chromosome carrying 18A5 (and a forked transgene inserted at cytological position 52 (provided by F. Díez-Benjumea).

In Situ Hybridizations

In situ hybridizations to whole-mount embryos using DNA or RNA probes were performed as described (Affolter et al., 1994; Hauptmann and Gerster, 1994).

To test whether the sequences corresponding to our putative shn cDNAs were transcribed in homozygous shn· embryos, the shn· chromosome was crossed over a CyO balancer chromosome containing an enhancer trap insert in the w gene (see Figure 1A). Embryos from this strain were collected and first incubated and stained to reveal anti-β-gal antibody distribution. Subsequently, these embryos were used for an in situ hybridization with an RNA probe derived from the last 4.5 kb of cDNA 5N. All balancer-containing embryos showed strong hybridization signals in the pattern shown in Figure 5; no hybridization signal could be detected in homozygous shn· embryos.

Isolation of Genomic and cDNA Clones

Genomic phages covering the shn locus were isolated from an EMBL 4 library that was a gift of A. Preis. An embryonic 0–3 hr λgt10 library (provided by T. Kornberg and L. Kauvar) was screened with the entire XbaI rescue fragment. Positive clones were rescreened with a 1.6 kb genomic SpeI fragment 6.5 kb 3’ to the P element insertion, or a 0.8 kb HindIII fragment just adjacent to the P element insertion. These two fragments were chosen because they represented unique sequences in the Drosophila genome. Sequencing of the longest phage positive for the SpeI probe (7TK; see Figure 6A) revealed an open reading frame of 1.7 kb extending through the entire cDNA. There was no positive clone for the HindIII fragment. To get a larger collection of cDNA clones, a λZAPII imaginal disc library (Nellen et al., 1994) was screened with the same two genomic fragments. Two phages positive for the HindIII fragment were obtained, and the longer (B17) was entirely sequenced. No phages containing inserts longer than 7TK were identified with the SpeI probe. To identify longer cDNAs extending 7TK in the 5’ and 3’ direction, three other libraries were screened by using the genomic HindIII fragment and the phage 7TK insert as probes. The two longest of the multiple phages recovered from a λgt10 library (embryonic development 0–4 hr, provided by M. Noll) extended 5’ (10N) and 3’ (1N) of 7TK. None of the multiple clones recovered from the other λgt10 library (T. Kornberg) was longer than 7TK. From both libraries most phages were identified at least twice, indicating that the libraries were screened to saturation. Only from a λgt11 library (screen performed by S. Baumgartner) could additional clones positive for the HindIII probe be identified, however, they were contained within B17. In additional rounds of screening (λgt10 library, embryonic development 0–4 hr from M. Noll), the 5’-most sequences of available cDNAs were used to walk toward the transcription start site; this led to the isolation of 13N and 1N. cDNA B3 was isolated from the λZAPII imaginal disc library by using polymerase chain reaction (PCR) amplification with nested primers.

DNA sequence was determined by primer walking, or with the help of nested deletions, by dideoxy chain termination using either Sequenase (United States Biochemical Corporation) or AmpliTaq (Prism Dye-Dideoxy Terminator, Perkin-Elmer) on an ABI 373A stretch sequencer.
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