Regulation of a dpp target gene in the Drosophila embryo

Jannette Rusch and Michael Levine*

Department of Biology, Center for Molecular Genetics, Pacific Hall, 9500 Gilman Drive, UCSD, La Jolla, CA 92093-0357, USA

*Current address: Dept. MCB, Division of Genetics, 401 Barker Hall, University of California, Berkeley, CA 94720, USA

SUMMARY

In Drosophila, two TGF-β growth factors, dpp and screw, function synergistically to subdivide the dorsal ectoderm into two embryonic tissues, the amnioserosa and dorsal epidermis. Previous studies have shown that peak dpp activity is required for the localized expression of zerknüllt (zen), which encodes a homeodomain transcription factor. We present evidence that zen directly activates the amnioserosa-specific expression of a downstream target gene, Race (Related to angiotensin converting enzyme). A 533 bp enhancer from the Race promoter region is shown to mediate selective expression in the amnioserosa, as well as the anterior and posterior midgut rudiments. This enhancer contains three zen protein binding sites, and mutations in these sites virtually abolish the expression of an otherwise normal Race-lacZ fusion gene in the amnioserosa, but not in the gut. Genetic epistasis experiments suggest that zen is not the sole activator of Race, although a hyperactivated form of zen (a zen-VP16 fusion protein) can partially complement reduced levels of dpp activity. These results suggest that dpp regulates multiple transcription factors, which function synergistically to specify the amnioserosa.

Key words: Drosophila, dpp, TGF-β, screw, amnioserosa, epidermis, zerknüllt, transcription factor, Race

INTRODUCTION

The transforming growth factor β (TGF-β) superfamily has been implicated in a variety of developmental processes (reviewed by Kingsley, 1994; Wall and Hogan, 1994). In Drosophila, the best studied member of this family is encoded by the decapentaplegic (dpp) gene (Padgett et al., 1987). dpp participates in several developmental processes, including the subdivision of the dorsal ectoderm in the early embryo, induction of lateral mesoderm in midstage embryos, midgut morphogenesis in advanced-stage embryos, and the patterning and growth of wing, leg and eye imaginal discs in larvae (reviewed by Bienz, 1994; Massagué et al., 1994; Rusch and Levine, 1996).

Recent studies have identified a number of components in the dpp signaling pathway, including serine/threonine receptor kinases encoded by the saxophone, thick veins and punt genes (Brummel et al., 1994; Nellen et al., 1994; Penton et al., 1994; Xie et al., 1994; Letsou et al., 1995; Ruberte et al., 1995). The focus of the present study concerns the characterization of dpp target genes expressed in the early embryo. Previous studies have identified target genes in the lateral mesoderm of midstage embryos (Staehling-Hampton et al., 1994; Frasch, 1995), the midgut of advanced stage embryos (reviewed by Bienz, 1994) and the imaginal discs of third-instar larvae (Lecuit et al., 1996; Nellen et al., 1996). Sustained expression of the MADS-containing gene, tinman (tin), in the lateral mesoderm and heart depends on induction by dpp in the dorsal ectoderm (Staehling-Hampton et al., 1994; Frasch, 1995). In the embryonic midgut, dpp emanating from the visceral mesoderm triggers the underlying endoderm to activate the expression of the homeobox-containing homeotic selector gene labial (reviewed by Bienz, 1994). Minimal labial enhancers have been identified that mediate this induction and it has been suggested that the labial protein functions synergistically with an additional, unknown factor which is stimulated in response to dpp signaling. dpp is also part of an autoregulatory loop, whereby the homeotic selector gene Ultrabithorax (Ubx) activates dpp in the visceral mesoderm and then dpp reinforces Ubx expression (Bienz, 1994). In wing discs, dpp signaling activates the expression of two genes that encode putative sequence-specific transcription factors, optomotor-blind and spalt (Lecuit et al., 1996; Nellen et al., 1996). Despite these advances, the identities of the transcription factors that mediate dpp signaling remain elusive.

Our focus has been on the earliest dpp-mediated patterning process, the subdivision of the dorsal ectoderm. In pregastrula embryos, dpp is responsible for specifying two different tissues along the dorsal-ventral (DV) axis, the amnioserosa and dorsal epidermis. dpp− embryos lack both tissues and the analysis of cuticle preparations suggest that they are transformed into neuropoderm, so that ventral denticle belts encircle the entire circumference of the embryo (Irish and Gelbart, 1987). Weaker dpp mutant alleles result in the transformation of the amnioserosa into dorsal epidermis. Genetic studies (Ferguson and Anderson, 1992a; Arora et al., 1994) and embryo injection assays (Ferguson and Anderson, 1992b) prompted the proposal that a dpp activity gradient is distributed along the DV axis of the early embryo. Peak levels of dpp activity in the dorsal-most regions of the embryo specify the amnioserosa. Lower levels of dpp specify the dorsal epidermis in dorsolateral and lateral regions. Recent studies suggest that dpp acts in concert with...
screw (scw), another TGF-β homologue, to specify the amnioserosa (Arora et al., 1994).

Here, we investigate the regulation of Race (Related to angiotensin converting enzyme), which is the earliest known marker gene for the amnioserosa (Tatei et al., 1995). Genetic studies suggest that Race may be a direct target of the dpp signaling pathway. Previous studies have shown that peak levels of dpp activity are required for sustained expression of the homeobox gene zerknullt (zen) in the differentiating amnioserosa (Ray et al., 1991). We present evidence that zen directly activates Race expression. A minimal, 533 bp enhancer from the Race promoter region mediates expression in the amnioserosa, as well as the anterior and posterior midgut rudiments. This enhancer contains three zen protein binding sites, and mutations in these sites selectively eliminate the expression of a Race-lacZ fusion gene in the amnioserosa, but not in the gut. These experiments establish a regulatory link between zen and Race. Genetic epistasis tests suggest that high levels of dpp activity can partially complement zen embryos, suggesting that dpp regulates multiple transcription factors in the presumptive amnioserosa. A hyperactivated form of zen (a zen-VP16 fusion protein) can partly compensate for reduced levels of dpp activity. We propose that the dpp signaling pathway regulates multiple transcription factors, which function synergistically to specify the amnioserosa.

MATERIALS AND METHODS

Drosophila stocks

The fly stocks used were: parental line for P-transformation, yw67C23, zerknullt, zen30/TM3(frt-lacZ) (Wakimoto et al., 1984); tolloid, tkp666/TM3 (Jürgens et al., 1984); decapentaplegic, dpp1013/Gia Dp(2;2)DTD48 (Ray et al., 1991); screw, scw12 (obtained from K. Arora); saxophone, sax1 and sax2 (obtained from E. Wieschaus); thick veins, FRT40A-ksv12 (obtained from K. Basler); schnurri, shn223 (Arora et al., 1995).

The procedure of Chou and Perrimon (1992) was used to produce schnurri (shn) mutant germ-line clones. Males carrying the FRT42B-ovoB1 chromosome (Chou et al., 1993) were mated with FRT42B-shn12/CyO females (Arora et al., 1995), which also contain the hsFLP1 chromosome [w1118[Prv1+hsFLP1]]. Fi progeny were heat shocked for 2 hours at 37°C at the larval third instar stage and allowed to develop at 25°C. Virgin FRT-shn12/0 females were then mated with FRT-shn12/CyO males, and the resulting embryos were collected and analyzed.

Epistasis tests with the zenVP16 transgene were done by standard genetic methods. For example, the transgene was recombined onto the sax1 chromosome and the resulting stock was mated with the sax1 strain, sax1, P(zenVP16)sax1 females were selected and their progeny collected for staining. All embryos were collected and aged at 25°C.

Plasmids

The dpp-bcd transgene was made by creating an Ndel site at the initiating codon of the dpp coding sequence in the pKS+ vector (Stratagene). A 1.9 kb Ndel-Sspl fragment was then bluntly into the HindIII and Spl sites of a pKS+ vector containing the 1 kb fragment of the maternally expressed hsp83 promoter (Xiao and Lis, 1989). A KpnI+SacI fragment containing the hsp83 promoter and the dpp coding sequence was then ligated with a 0.9 kb SacII-Xbal fragment containing the bicoid 3' UTR (Macdonald and Struhl, 1988).

The zen and scw transgenes were made in similar ways. For zen, a 1.2 kb Ndel-Sspl fragment from pAR-zen (Hoey and Levine, 1988) was used. For scw, an Ndel site was created at the start codon of the coding sequence, and an internal Ndel site at pos 167 of the cDNA was eliminated, both by site directed mutagenesis in pKS+. A 1.3 kb Ndel-Dral fragment was then used for cloning into pKS+-hsp83. The pKS+-scw plasmid was a gift from K. Arora (Arora et al., 1994).

The dFOS transgene was prepared with a 2 kb Ndel fragment from pAR-dFRA, which was bluntly with Klenow enzyme and ligated into pKS+-hsp83. The plasmid pAR-dFRA, containing the Drosophila fos coding sequence was a gift from R. Tjian (Perkins et al., 1990).

The zenVP16-bcd transgene was prepared with a 237 bp fragment containing codons 413-490 of VP16, which was amplified by PCR from plasmid pML2 (obtained from J. T. Kadonaga; Triebenzer, 1995), using primers containing a BamHI and a SacI restriction site, and cloned into pKS+. The stop codon of the zen coding sequence in pKS+-hsp83-zen was eliminated by site-directed mutagenesis and a Smal site was created. This also eliminated the last codon of the zen coding sequence (Asn). After blotting the BamHI site of pKS+-VP16, the 237bp BamHI-SacII VP16 fragment was ligated into the mutagenized pKS+-hsp83-zen Smal plasmid. A 2.2 kb KpnI-SacII fragment containing the hsp83 promoter and the zenVP16 fusion was then triple ligated into pCaSpeR as described above. For the constructs containing the tld promoter, a 0.7Mbp BamHI-EcoRI fragment from pCaSpeR-tild was subcloned into pKS+. This was then cloned into pCaSpeR-AUGgla as a BamHI-KpnI fragment. Subsequently, coding sequences were inserted into the KpnI and Xhol sites.

The pCaSpeR-tld plasmid contains the proximal 700 bp from the tld promoter region (Kirov et al., 1994; provided by M. O’Connor). The pCaTdzen fusion gene was prepared with a 1.2 kb Apol fragment from pKS+-hsp83-zen. This was first subcloned into the EcoRI site of pKS+, bringing along 85 bp from the 3' end of the hsp83 promoter with the zen coding sequence. A KpnI-Xhol fragment was then ligated into pCa-tld. The pCaTdzenVP16 transgene was prepared with a 1.4 kb Apol-SacII fragment from pKS+-hsp83-zenVP16, which was cloned into the EcoRI and Smal sites of pKS+, after blunting the SacII site. This fragment, which also included 85 bp from the 3' end of the hsp83 promoter, was then ligated into pCa-tld, using the KpnI and Xhol sites.

Race-lacZ fusion genes were prepared with a 533 bp Clal-Xhol fragment mapping between −2 kb and −1.5 upstream of the Race transcription start site. The fragment was bluntly with Klenow enzyme, and cloned into the Norl site of pKS+ using Norl linkers. Both strands were sequenced using the Sequenase kit (US Biochemical). The zen fusion gene, which lacks zen protein binding sites, was prepared by site-directed mutagenesis, using the following oligonucleotides:

- GGGAGAATAGTCGCCCGGTATTTTTCATCAAGACG
- GAGAAGTGTACGOACAGGCAGCAGATCCTCACAAGCG
- GCGGCTACCTGCGCCAGAAATACAGCGGCTGAGCCGCGC

(nucleotide substitutions are underlined). Mutagenized Norl fragments were then ligated into the blunt EcoRI site of −2evelacZCaSpeR using Norl linkers. The pCaSpeR derivative −2evelacZCaSpeR contains the basal promoter from the even-skipped gene and is described in Small et al. (1992).

P-element plasmids were injected into the yw67C23 strain together with the Δ2,3 transposase helper plasmid. For each P-element transgene, at least three independent transformant lines were analyzed. In situ hybridization assays were done as described previously using digoxigenin-UTP-labeled antisense riboprobes (Tautz and Pflieder, 1989; Jiang et al., 1991). Stained embryos were photographed using Nomarski optics.

zen protein purification and DNA-binding assays

A full-length zen protein was prepared with the T7 expression vector pAR3040 (Hoey and Levine, 1988), in the bacterial strain BL21(DE3) as described in Studier and Moffatt (1986). Induced cells were harvested by centrifugation and resuspended in 2.5mI of lysis buffer (50 mM Tris pH 8.0, 50 mM NaCl, 5 mM EDTA, 7.5 mM β-mercaptoethanol). After
addition of 5 mg of lysozyme and incubation for 15 minutes at RT, the cells were frozen in liquid nitrogen and thawed at room temperature. Urea lysis buffer (8 M urea, 1 M NaCl, 50 mM Tris pH 8.0, 7.5 mM β-mercaptoethanol) was added to a final concentration of 3 M urea. The supernatant was then centrifuged for 1 hour at 35,000 revs/minute at 10°C. After addition of 10% glycerol, the supernatant was aliquoted into 10 mM Tris pH 8.0, 1 mM EDTA, followed by EtOH precipitation. For each reaction, 250,000 counts/minute of labeled DNA was used. The footprinting procedure was done according to a protocol used by the Kadonaga lab. Binding reactions were done by incubating 25 μl of probe mix (containing 4% polyvinyl alcohol and 1 mg of poly d(I-C) labeled with Klenow and redigested with BbsI site of pKS+). Plasmids were digested with EcoRI, 32P-labeled with Klenow and redigested with BamHI. The labeled fragments were isolated from a 5% acrylamide gel by soaking the crushed gel slice in 10 mM Tris pH 8.0, 1 mM EDTA, followed by EtOH precipitation. For each reaction, 250,000 counts/minute of labeled DNA was used. The footprinting procedure was done according to a protocol used by the Kadonaga lab. Binding reactions were done by incubating 25 μl of probe mix (containing 4% polyvinyl alcohol and 1 mg of poly d(I-C)) with 25 μl of protein diluted in buffer Z (25 mM Hepes pH 7.5, 12.5 mM MgCl2, 20% glycerol, 0.1% NP-40, 10 mM ZnSO4, 1 mM DTT) for 15 minutes on ice. The final concentration of KCl in the mix was 50 mM. For reactions containing up to 5 μl protein, the 2.5 mg/ml DNasel stock (Worthington) was diluted 1:1000 in ice-cold water, for 10 or 20 μl protein, it was diluted 1:500. To each sample, 50 μl of 10 mM MgCl2, 5 mM CaCl2, and 2 μl of the diluted DNasel was added. After 1 minute, the reaction was stopped by addition of 90 μl stop solution (20 mM EDTA pH 8.0, 1% SDS, 0.2 M NaCl, 250 mg/ml glycogen). Following a 5 minutes incubation with 10 μl of 2.5 mg/ml proteinase K (Boehringer), the samples were phenol-chloroform extracted, EtOH precipitated and the pellets were dissolved in formamide loading buffer. For all three fragments, both strands were footprinted; for the opposite strand, the footprinting procedure was done according to a protocol used by the Kadonaga lab. Binding reactions were done by incubating 25 μl of probe mix (containing 4% polyvinyl alcohol and 1 mg of poly d(I-C)) with 25 μl of protein diluted in buffer Z (25 mM Hepes pH 7.5, 12.5 mM MgCl2, 20% glycerol, 0.1% NP-40, 10 mM ZnSO4, 1 mM DTT) for 15 minutes on ice. The final concentration of KCl in the mix was 50 mM. For reactions containing up to 5 μl protein, the 2.5 mg/ml DNasel stock (Worthington) was diluted 1:1000 in ice-cold water, for 10 or 20 μl protein, it was diluted 1:500. To each sample, 50 μl of 10 mM MgCl2, 5 mM CaCl2, and 2 μl of the diluted DNasel was added. After 1 minute, the reaction was stopped by addition of 90 μl stop solution (20 mM EDTA pH 8.0, 1% SDS, 0.2 M NaCl, 250 mg/ml glycogen). Following a 5 minutes incubation with 10 μl of 2.5 mg/ml proteinase K (Boehringer), the samples were phenol-chloroform extracted, EtOH precipitated and the pellets were dissolved in formamide loading buffer. For all three fragments, both strands were footprinted; for the opposite strand, the BamHI site was labeled with 32P, followed by digestion with EcoRI.

RESULTS

Regulation of Race expression

Seven zygotically active genes are required for the specification of the amnioserosa. Besides dpp and zen, this group includes tolloid (tld), screw (scw), twisted gastrulation (tsg), short gastrulation (sog) and shrew (srl) (reviewed by Rusch and Levine, 1996). Race expression is disrupted in each of the mutants tested and some examples are presented in Fig. 1. As shown previously (Tatei et al., 1995), the amnioserosa-specific Race expression pattern is absent in zen probe (Fig. 1B). The same result was obtained in scw, tld and tsg mutants (data not shown). In all these cases, Race expression is unaffected in the AMG and PMG (data not shown).

The early Race expression pattern is lost in mutants that contain reductions in the dpp activity gradient. For example,
**Race is a zen target gene**

To determine whether *zen* regulates Race expression, we characterized 5′ sequences from the Race promoter region in transgenic embryos (see Materials and Methods). This involved attaching various DNA fragments from the Race promoter region to a *lacZ* reporter gene. A Race-*lacZ* fusion gene containing the first 4.4 kb of 5′ flanking sequence was found to be expressed in both the amnioserosa and gut rudiments in transgenic embryos, similar to the endogenous Race pattern (data not shown). Fusion genes containing either 2.4 kb or 2.0 kb of 5′ flanking sequences also directed a normal Race expression pattern; however, truncations containing 1.5 kb, 1.1 kb, 0.8 kb or 0.5 kb of 5′ flanking sequence failed to direct detectable expression. These experiments suggested that the region between −2.0 kb and −1.5 kb was essential for the regulated pattern of Race expression.

A 533 bp *ClaI*-XhoI fragment, located between −2.0 kb and −1.5 kb upstream of the Race transcription start site, was found to be sufficient to direct expression of a *lacZ* reporter gene in a pattern that closely resembles the endogenous Race gene. This Race-*lacZ* transgene is initially expressed in the presumptive amnioserosa (Fig. 2A). Staining persists in older embryos (Fig. 2B; arrow), expression is also detected in the AMG and PMG during germ band elongation (Fig. 2B; arrowheads). The early pattern (Fig. 2A) is lost when the transgene is crossed into *zen*− mutants; in contrast, expression in the AMG and PMG is unaffected (not shown).

**Ectopic induction of the dpp pathway**

The preceding results suggest that *zen* is an essential activator of Race expression. Additional experiments were done to determine whether it is the sole activator of Race. Previous studies on embryonic enhancers, such as those mediating stripes and bands of gene expression, suggest that they are regulated by multiple activators (reviewed by Hoch and Jäckle, 1993; Gray and Levine, 1996).

Genetic epistasis tests were done to determine whether high levels of *dpp* activity can complement *zen*− mutants. These experiments involved misexpressing *dpp* in anterior regions of early embryos. A fusion gene was prepared that contains the *dpp* coding sequence attached to the 3′ untranslated region (UTR) of *bicoid*, which has been shown to be sufficient for the localization of heterologous transcripts to the anterior pole of developing oocytes and early embryos (Macdonald and Struhl, 1988). This *dpp*-bcd fusion gene was placed under the control of a 1 kb fragment from the promoter region of *hsp83*, which directs maternal expression during oogenesis (Xiao and Lis, 1989). Transgenic embryos exhibit ectopic *dpp* expression in anterior regions (Fig. 4A). This maternal transcript diminishes during cleavage cycle 14 and is lost by the completion of cellularization (not shown). The levels of ectopic *dpp* transcripts appear to be several-fold higher than the endogenous *dpp* mRNA. These ectopic transcripts result in the activation of Race in anterior regions (Fig. 4B; arrow; the arrowhead identifies the endogenous Race pattern). Similar experiments with *scw* do not result in the ectopic activation of Race (Fig. 4C,D). These experiments highlight the importance of *dpp* in DV patterning and are consistent with previous epistasis tests, which suggest that *scw* functions by augmenting *dpp* activity (Arora et al., 1994).

We analyzed the consequences of expressing the *dpp* transgene in various mutants, including *zen*− embryos (Fig. 5). The requirement for endogenous *dpp* was tested by crossing the transgene into *dpp*− embryos (Fig. 5A). Ectopic Race expression is observed in anterior regions, even though the endogenous Race pattern is eliminated (see Fig. 4B). Similar Race expression patterns are also observed in *tdl*− (Fig. 5D) and *tsg*− (data not shown) mutant embryos. The former result is consistent with the previous demonstration that doubling the *dpp* gene copy number suppresses weak *tdl* mutant alleles (Ferguson and Anderson, 1992a). Similarly, the *dpp* transgene activates Race expression in *scw*− embryos (Fig. 5C), which is consistent with RNA injection assays whereby high concentrations of *dpp* RNA can induce the differentiation of amnioserosa in *scw*− embryos (Arora et al., 1994).

Previous epistasis tests did not examine the possibility that increased levels of *dpp* activity might complement *zen* mutants. Expression of the *dpp* transgene in *zen*− embryos results in the ectopic activation of Race in anterior regions (Fig. 5B), although, as in the case of *scw* mutants, there is a reduction in the levels of expression (compare with Fig. 5D). These results strongly suggest that *zen* is an essential, but not sole, mediator of the *dpp* pathway.

**Hyperactivation of zen can partially complement reductions in dpp signaling**

The preceding results suggest that *zen* is necessary but not sufficient for the activation of Race. Further support for this view was obtained by analyzing the activities of an hsp83-*zen-bcd
transgene, similar to the dpp and scw transgenes described previously. Ectopic expression of zen at the anterior pole (Fig. 6A) is not sufficient to induce Race (Fig. 6B).

The simplest explanation for these findings is that dpp regulates multiple transcription factors, including zen, which in turn specify the amnioserosa through the activation of Race and other downstream target genes. Previous studies have shown that localized patterns of gene expression often depend on synergistic interactions between different classes of transcriptional activators. For example, bicoid and hunchback are both required for the activation of the eve stripe 2 enhancer (Small et al., 1991). Disruptions in bicoid-hunchback synergy can be compensated by hyperactivated forms of bcd (a bcd-GCN4 fusion protein; Arnosti et al., 1996). To determine whether similar mechanisms might apply to the regulation of Race, we examined the activities of a hyperactivated form of zen.

This was accomplished by fusing the heterologous VP16 activation domain to the zen coding sequence. Unfortunately, the resulting zenVP16 chimera appears to be toxic and, consequently, relatively few transgenic lines were obtained. Moreover, the established lines express only very low levels of the zenVP16 mRNA (data not shown). Nonetheless, the chimeric protein induces ectopic expression of Race (Fig. 6C), whereas substantially higher levels of the normal zen protein fail to activate Race (Fig. 6B).

Additional experiments suggest that the zenVP16 fusion protein not only activates Race, but is able to complement reductions in the dpp signaling pathway. The zenVP16 transgene was crossed into embryos derived from sax1/sax2 trans heterozygous females. Such mutants lack the normal amnioserosa-specific Race expression pattern (Fig. 1C), indicating a significant reduction in dpp signaling. Despite the loss of amnioserosa in these mutants, the chimeric zenVP16 protein continues to mediate ectopic activation of Race (Fig. 6D, compare with 6C).

A more critical genetic complementation experiment involved expressing the zenVP16 chimeric protein in dorsal regions of dpp+/+ heterozygotes (Fig. 7). As discussed previously, embryos carrying a single copy of dpp exhibit severe reductions in the amnioserosa. Race expression is nearly lost
D. (A) -bcd transgene in a dpp that gives weaker expression of embryos in A and C were derived from a transgenic line transgene, and are staged and oriented as in Fig. 2. The only the endogenous pattern is detected. with a Race hybridization probe. No ectopic staining is observed; embryo carrying the ectopic expression is observed at the anterior pole. (D) Cellularizing wild-type embryos.

-bcd transgene in a dpp (C) wild-type embryos. The endogenous Race pattern is gone. activation is reduced by 2 or 3-fold as compared with

Ectopic activation of Race. Embryos were hybridized with a Race probe and oriented as in Fig. 2. (A) Nuclear cycle 13 embryo derived from a wild-type female carrying the dpp-bcd transgene. The embryo was hybridized with a dig-U-labeled dpp antisense RNA probe. Transcripts are localized in anterior regions, due to the bicoid 3’ UTR. (B) Nuclear cycle 14 embryo carrying the dpp-bcd transgene. The embryo was stained with a Race hybridization probe. Strong ectopic activation of Race (arrow) extends as an anteroposterior gradient and overlaps with the endogenous Race pattern in the presumptive amnioserosa (arrowhead). (C) Nuclear cycle 10/11 embryo carrying a scw-bcd transgene. The embryo was stained with a scw hybridization probe. As for the dpp transgene, ectopic expression is observed at the anterior pole. (D) Cellularizing embryo carrying the scw-bcd transgene. The embryo was stained with a Race hybridization probe. No ectopic staining is observed; only the endogenous pattern is detected.

in these embryos (Fig. 7B, compare with 7A). The zen-VP16 chimeric coding sequence was placed under the control of the tld promoter in an effort to determine whether the constitutively activated protein can restore some of the dorsal tissues lost in dpp+/ heterozygotes. A 700 bp fragment from the proximal tld promoter region was shown to direct expression in dorsal tissues, even in dpp mutants, thereby establishing tld as one of the few DV patterning genes whose expression does not depend on the dpp signaling pathway (Kirov et al., 1994). This tld regulatory DNA was used to drive the expression of the zenVP16 fusion gene, as well as the normal zen coding sequence.

Transgenic embryos were hybridized with zen antisense RNA probes to make certain that the tld promoter directs efficient expression in dorsal tissues (data not shown). Both transgenes, tld-zen and tld-zenVP16, were analyzed for Race expression in dpp heterozygous embryos (Fig. 7C,D). The normal zen gene is not very effective in augmenting the number of Race-expressing cells in the differentiating amnioserosa (Fig. 7C; compare with B). In contrast, the zenVP16 transgene restores a substantial number of Race-positive cells (Fig. 7D). Moreover, the stained cells have the appearance of differentiating amnioserosa cells, in that they exhibit a squamous morphology and possess enlarged nuclei. These results suggest that the zen-VP16 fusion protein can complement reductions in dpp signaling.

**DISCUSSION**

We have presented evidence that the dpp signaling pathway activates Race expression in the presumptive amnioserosa via zen. Genetic epistasis tests were conducted using a dpp-bcd fusion gene that is expressed in an anteroposterior gradient in the early Drosophila embryo. These tests are consistent with previous studies suggesting that most of the zygotic DV patterning genes participate in the establishment of a dpp activity gradient. The finding that the dpp-bcd transgene mediates ectopic activation of Race in zen- mutants suggests that dpp specifies the amnioserosa through the regulation of multiple transcription factors. This view is supported by the demonstration that a hyperactivated form of zen only partially complements reductions in dpp signaling. We propose that the dpp pathway regulates multiple factors, which function synergistically to specify the amnioserosa.

**The Race enhancer**

Previous studies on signaling pathways have emphasized the identification of individual transcription factors in the regulation of downstream target genes. For example, the Toll/IL-1 signaling pathway regulates Rel-domain transcription factors, such as dorsal and NF-kB (Wasserman, 1993). The Notch pathway modulates the suppressor of Hairless (su(H)) activator (Fortini and Artavanis-Tsakonas, 1994; Bailey and Posakony, 1995), while recent studies suggest that the cubitus interruptus zinc
Regulation of a *dpp* target gene in *Drosophila*

**Fig. 6.** Hyperactivated form of the zen protein. Embryos are oriented as in Fig. 2. (A) Nuclear cycle 10/11 embryo carrying a *zen*-bcd transgene. Hybridization with a *zen* probe reveals weak expression in anterior regions. This staining is weaker than that observed for the *dpp*-bcd and *sce*-bcd transgenes (see Fig. 4A,C). (B) Nuclear cycle 14 embryo carrying the *zen*-bcd transgene. The embryo was stained with a Race hybridization probe. There is no detectable ectopic expression and endogenous Race is not yet activated. (C) Nuclear cycle 14 embryo carrying a *zen*VP16-bcd transgene. The embryo was stained with a Race hybridization probe. There is weak ectopic activation of Race. (D) Same as C, except that the *zen*VP16-bcd transgene was crossed into an embryo derived from a *sax*1/sax2 transheterozygous female. Ectopic activation of Race is unaffected by the *sax* mutations.

**Fig. 7.** Genetic complementation assays with the *zen*-VP16 transgene. Embryos are oriented with anterior to the left and tilted to give a dorsolateral view of the amnioserosa. All embryos are at the rapid phase of germband elongation and stained for Race expression. (A) Wild-type embryo. Strong Race staining of the amnioserosa, which is characterized by a squamous morphology with enlarged nuclei (see arrow). (B) *dpp* heterozygote. There is a severe reduction of the amnioserosa and Race staining pattern. Expression is nearly lost in the region corresponding to the amnioserosa (arrowhead). (C) *dpp* heterozygous embryo carrying the tld-*zen* fusion gene. There are only a few Race-positive cells in the region corresponding to the amnioserosa (arrowhead). For most transgenic lines, the embryos are indistinguishable from *dpp* heterozygotes carrying no transgene (see B). (D) *dpp* heterozygote carrying the tld-*zen*VP16 fusion gene. The number of cells expressing Race is significantly increased in dorsal regions (arrowhead). The stained cells display the squamous morphology and enlarged nuclei typical for amnioserosa cells (compare to A).

---

The identities of these additional regulatory factors? Recent genetic screens have identified the zinc finger protein encoded by the *schnurri* (*shn*) locus as a potential target of the *dpp* pathway (Arora et al., 1995; Grieder et al., 1995; Staehling-Hampton et al., 1995). However, endogenous Race expression was found to be normal in embryos derived from females carrying *shn* mutant germ-line clones, suggesting that *shn* is dispensable for the specification of the amnioserosa and other aspects of early DV patterning (J. Rusch, unpublished results).

It is possible that dFOS represents a second target of the *eve* stripe 2 enhancer is activated by bicoid and hunchback, while the *rho* enhancer is activated by dorsal and twist.

This study has identified *zen* as an important mediator of the *dpp* pathway in the specification of the amnioserosa. A 533 bp enhancer from the *Race* promoter region mediates expression in the amnioserosa, as well as the AMG and PMG. The amnioserosa-specific pattern is selectively lost in *Race-lacZ* fusion genes that contain mutations in the three *zen* protein binding sites identified by DNaseI footprint assays. This result suggests a direct regulatory link between *zen* and *Race*, but does not exclude the possibility that additional transcription factors are also important for Race expression. For example, mutations in either dorsal or twist activator sites result in a severe impairment of the *rho* enhancer (Ip et al., 1992).

**dpp regulates multiple transcription factors**

*zen* does not appear to be the sole mediator of *dpp* signaling in the amnioserosa. A *dpp*-bcd transgene mediates ectopic induction of Race expression in anterior regions of early embryos lacking *zen*+ gene activity. This transgene also mediates weak activation of a defective *Race-lacZ* fusion gene lacking the three *zen* protein binding sites (J. Rusch, data not shown). Moreover, a hyperactivated form of *zen* (*zen*-VP16) only partially restores Race expression in mutant embryos carrying reductions in *dpp* signaling. The simplest interpretation of these results is that *dpp* regulates at least one additional transcription factor, which functions synergistically with *zen* to activate Race and other downstream target genes in the presumptive amnioserosa.

What are the identities of these additional regulatory factors? Recent genetic screens have identified the zinc finger protein encoded by the *schnurri* (*shn*) locus as a potential target of the *dpp* pathway (Arora et al., 1995; Grieder et al., 1995; Staehling-Hampton et al., 1995). However, endogenous Race expression was found to be normal in embryos derived from females carrying *shn* mutant germ-line clones, suggesting that *shn* is dispensable for the specification of the amnioserosa and other aspects of early DV patterning (J. Rusch, unpublished results).

It is possible that dFOS represents a second target of the
Fig. 8. Summary of dpp-mediated gene expression in the presumptive amnioserosa. dpp and scw activate one or more receptor serine/threonine kinase complexes in the dorsal-most regions of the early embryo. This results in the activation of multiple transcription factors, including zen and X (possibly dFOS). It is currently unclear whether these factors serve as substrates for the dpp signaling pathway. Perhaps an unknown, pre-existing factor is modified by the pathway, and then helps activate zen and X expression. These latter proteins bind to the Race promoter region, and possibly to the promoter regions of additional amnioserosa-specific target genes (AS). zen and X activate these target genes through some type of transcriptional synergy, possibly cooperative occupancy of linked operator sites.

dpp pathway. dFOS is maternally expressed throughout the early embryo (Perkins et al., 1990). Zygotic expression of the gene is restricted to the presumptive amnioserosa prior to the completion of cellularization (J. Rusch, unpublished results). The 533 bp Race enhancer contains several potential AP-1/dFOS binding sites; three of these are closely linked to zen binding sites (see Fig. 3B). Moreover, a Drosophila-bcd transgene mediates transient ectopic activation of zen, suggesting a regulatory link between zen and dFOS (J. Rusch data not shown). These observations raise the possibility that zen and dFOS function synergistically to activate Race and other amnioserosa-specific target genes. Previous studies have shown that a minimal labial enhancer, which is induced by dpp in the midgut, contains putative CRE recognition sites (Tremml and Bienz, 1992; M. Bienz, personal communication). AP-1 and CRE sequences are related (TGANTCA and TGANNTCA, respectively) and bind different members of the bZIP family of transcription factors, which include JUN/FOS and CREB. Perhaps Hox-bZIP synergy is a common feature of dpp signaling.

It is possible that the dpp pathway leads to the post-translational modification of either zen or dFOS since both proteins are present in early embryos, prior to the time when dpp is active. However, it is equally likely that an unknown transcription factor serves as a dpp substrate and then participates in the activation of zen and dFOS. Given the early stages when the dpp-bcd transgene induces ectopic Race expression, such a putative unknown factor should probably be maternally expressed.

dpp haploinsufficiency

dpp is one of just a small number of genes in the Drosophila genome that exhibits haploinsufficiency (Ray et al., 1991). dpp/+ heterozygotes exhibit a severe reduction in the amnioserosa and die during embryogenesis. We propose that this extreme dosage sensitivity stems from synergistic activation of Race and other amnioserosa-specific target genes, as summarized in Fig. 8. According to this view, dpp is expressed at limiting amounts in early embryos. Slight reductions in dpp activity might cause a severe breakdown in the activation of amnioserosa-specific target genes due to the loss of zen-X synergy. It is conceivable that this synergy involves cooperative binding to DNA since the zen recognition sequences present in the Race enhancer are quite divergent from the optimal sites identified previously (Hoey and Levine, 1988).

This model for dpp haploinsufficiency is reminiscent of mesoderm specification and sex determination in Drosophila. Embryos derived from dll/+ heterozygous females exhibit a high incidence of lethality at elevated temperatures (Simpson, 1983). This lethality is associated with reductions in mesodermal tissues (Kosman et al., 1991; Gonzalez-Crespo and Levine, 1993). Mesoderm-specific target genes, such as snail, are activated by a combination of dorsal and bHLH proteins, such as twist. Reductions in dorsal lead to reduced levels of twist protein and severe disruptions in the activation of snail due to a breakdown in dorsal-twist synergy (Ip et al., 1992). A similar failure in activator synergy appears to be the basis for sex determination in Drosophila (reviewed by Cline, 1993). Individuals with one copy of the X chromosome fail to activate the early Sex lethal promoter due to reductions in two key transcriptional activators, the bZIP sisA protein and the bHLH scute protein.

We thank Bill McGinnis, Jim Posakony and Ethan Bier for critically reading the manuscript. This work was funded by a grant from the NIH (GM 46638).

REFERENCES


