Peak levels of BMP in the *Drosophila* embryo control target genes by a feed-forward mechanism

Mu Xu, Nikolai Kirov and Christine Rushlow*

Department of Biology, New York University, 100 Washington Square East, New York, NY 10003, USA
*Author for correspondence (e-mail: chris.rushlow@nyu.edu)

Accepted 31 January 2005

Development 132, 1637-1647
Published by The Company of Biologists 2005
doi:10.1242/dev.01722

Summary

Gradients of morphogens determine cell fates by specifying discrete thresholds of gene activities. In the *Drosophila* embryo, a BMP gradient subdivides the dorsal ectoderm into amnioserosa and dorsal epidermis, and also inhibits neuroectoderm formation. A number of genes are differentially expressed in response to the gradient, but how their borders of expression are established is not well understood. We present evidence that the BMP gradient, via the Smads, provides a two-fold input in regulating the amnioserosa-specific target genes such as *Race*. Peak levels of Smads in the presumptive amnioserosa set the expression domain of *zen*, and then Smads act in combination with Zen to directly activate *Race*. This situation resembles a feed-forward mechanism of transcriptional regulation. In addition, we demonstrate that ectopically expressed Zen can activate targets like *Race* in the presence of low level Smads, indicating that the role of the highest activity of the BMP gradient is to activate *zen*.

Key words: BMP gradient, Zen, *Race*, Feed-forward

Introduction

A primary mechanism for establishing cell fates during animal development is to use morphogen gradients. A single gradient formed across a broad field of cells can determine a number of different cell types by generating thresholds for downstream target gene responses. For example, it is thought that the peak level of a gradient regulates a specific set of target genes, whereas lower levels regulate an additional set of genes.

In *Drosophila*, patterning of the embryonic dorsoventral axis depends on the combined action of two morphogens: Dorsal (Dl) and Decapentaplegic (Dpp). Dl is a maternally loaded transcription factor that is responsible for setting up the overall dorsoventral axis of the embryo (reviewed by Stathopoulos and Levine, 2002). A gradient of Dl protein is formed during early embryogenesis with peak levels in the ventral nuclei. The gradient acts to subdivide the axis into three main regions – ventral (mesoderm), lateral (neuroectoderm) and dorsal ectoderm – by eliciting several threshold responses from batteries of zygotic patterning genes. For example, transcriptional activation of *twist* (*twi*) and *snail* (*sna*) require high levels of Dl, while *short gastrulation* (*sog*), *brinker* (*brk*) and *rhomboid* (*rho*) can be activated by lower levels of Dl. Genes such as *zerknüllt* (*zen*), *decapentaplegic* (*dpp*) and *tolloid* (*tld*) are repressed by Dl and thus come to be expressed only in the dorsal region. Differential target gene responses are mediated largely by the affinity of Dl-binding sites in the target enhancers (Jiang and Levine, 1993).

Dpp acts to further subdivide the dorsal domain into amnioserosa (the dorsal most region) and dorsal ectoderm, while also inhibiting neuroectoderm formation (Ferguson and Anderson, 1992a; Wharton et. al., 1993; Biehs et al., 1996). Dpp is a member of the TGFβ superfamily of ligands, which are most closely related to the BMPs (bone morphogenetic proteins), and signals through a pathway comprising the type I and type II serine-threonine kinase transmembrane receptors and the intracellular Smad proteins, Mother against Dpp (Mad) and Medea (Med) (reviewed by Raftery and Sutherland, 2003). Upon ligand binding and receptor activation, Mad is phosphorylated (PMad), thereby allowing translocation into the nucleus along with the co-Smad Medea. In the nucleus, Smads function as DNA-binding transcription factors (reviewed by Shi and Massagué, 2003).

Although *dpp* RNAs are evenly distributed across the dorsal region of the precellular embryo, the Dpp activity gradient takes shape during stage 5, as the embryo is undergoing cellularization (reviewed by Raftery and Sutherland, 2003). The gradient, which also includes a second BMP ligand Screw (Scw) (Arora et al., 1994), is formed through a dynamic process involving the secreted protein Sog, which emanates from the adjacent ventral region (Srinivasan et al., 2002). As Sog diffuses dorsally, it sequesters BMPs with the help of another secreted protein Twisted Gastrulation (Tsg) (Mason et al., 1994; Ross et al., 2001). This tripartite complex is thought to serve two purposes that have opposite effects on BMP signaling. First, it antagonizes BMP signaling by preventing BMP ligands from interacting with their receptors; and second, it promotes signaling by allowing the redistribution of BMPs to more dorsal regions (Holley et al., 1995; Shimmi and O'Connor, 2003). Tolloid (Tld), a metalloprotease localized in the dorsal region (Shimell et al., 1991), cleaves Sog, thereby releasing BMPs as active ligands. The net effect is a BMP gradient that can be visualized by detecting the output of the BMP pathway, the nuclear Smad proteins (reviewed by Raftery and Sutherland, 2003). Initially PMad is in a broad gradient

containing relatively low levels of protein. This develops into a steep step-wise gradient with increasingly high levels in a five- to six-cell-wide stripe along the dorsal midline, the presumptive amnioserosa, and lower levels in the three or four cells adjacent to either side of the stripe. In more lateral regions of the dorsal ectoderm, PMad protein is not detectable by antibody staining.

There are several candidate BMP target genes whose expression domains correlate with the stepwise PMad gradient. For example, *hindsight* (*hnt*; *peb* – FlyBase) (Yip et al., 1997) and *Race* (related to angiotensin-converting enzyme; *Ance* – FlyBase) (Tatei et al., 1995) are expressed specifically in the peak level PMad domain. *u-shaped* (*ush*) (Cubadda et al., 1997; Jazwinska et al., 1999b), *tail-up* (*tup*) (Thor and Thomas, 1997; Ashe et al., 2000) and *rhomboid* (*rho*) (Bier et al., 1990; Ross et al., 2001) are expressed more broadly in 12-14 cells encompassing the adjacent lower level PMad domain. *pannier* (*pnr*) (Ramain et al., 1993; Winick et al., 1993) is expressed in a broad domain covering about 36 cells (or 25% of the dorsal-ventral circumference), the border of which does not correlate with a clear domain of PMad activity. The mechanism underlying threshold responses to the BMP/Smad gradient is not fully understood.

A possible mechanism emerged from studies on Brk, a transcriptional repressor (Campbell and Tomlinson, 1999; Jazwinska et al., 1999a; Jazwinska et al., 1999b; Minami et al., 1999). As Dpp signaling represses *brk* expression, *brk* domains are largely complementary to *dpp* domains, which allows target genes to be transcribed in the *dpp* domains. In areas where *dpp* and *brk* overlap slightly, Smads and Brk may compete for DNA binding on target enhancers as Brk sites often overlap Smad sites (Kirkpatrick et al., 2001; Rushlow et al., 2001; Saller and Bienz, 2001). It has therefore been suggested that a Brk gradient, inverse to the Smad gradient, acts to spatially restrict target gene activation and consequently sets borders of expression (Jazwinska et al., 1999a; Ashe et al., 2000; Müller et al., 2003). However, this mechanism does not explain the threshold responses of those genes that are not Brk targets, such as *Race*. Alternatively, a mechanism involving differential binding site affinity for Smads may play a role in establishing their borders of expression. Indeed Wharton et al. (Wharton et al., 2004) used modified *Race* enhancers to demonstrate that it is possible to broaden expression domains by increasing the affinity of Smad-binding sites.

We demonstrate that *Race* activation requires the combined input of Smads and Zen. Zen-binding sites lie adjacent to Smad sites in the *Race* enhancer, and we show that Smads facilitate the DNA binding of Zen and that this requires protein interaction between Smads and Zen. As *zen* is regulated by peak levels of the BMP gradient and thus becomes expressed only in the dorsalmost region (Rushlow et al., 2001), the regulation of *Race* resembles a feed-forward loop whereby one regulator, BMP/Smad, controls a second regulator, Zen, and then both bind and activate a common target gene, *Race*. In addition, we tested the respective roles of Zen and Smads in setting the expression borders of *Race.* When *zen* was expressed ectopically, *Race* expression broadened to encompass both the high and low level regions of the BMP gradient. Thus, as long as Zen is present, low levels of BMP activity are sufficient to activate *Race*, indicating that the purpose of the peak of the BMP gradient is to set the Zen domain.

Materials and methods

Fly strains

dpp^{hr4} is a weak hypomorphic allele and dpp^{H46} is a null allele (Wharton et al., 1993). dpp^{hr4} was balanced over *SM6 eve-lacZ*, while dpp^{H46} was balanced over *CyO23, P[dpp$^+$]*, a chromosome that contains two copies of *dpp* (Wharton et al., 1993). 4X *dpp* embryos are of the genotype: *CyO23, P[dpp$^+$]/CyO23, P[dpp$^+$]* and were derived from the dpp^{H46}*/CyO23, P[dpp$^+$]* stock. sog^{SY06} is a strong hypomorphic *sog* allele (Ferguson and Anderson, 1992b) balanced over *FM7c, ftz-lacZ*. zen^{w36} is a null allele (Rushlow et al., 1987a) balanced over *TM3, ftz-lacZ* or *TM3, hb-lacZ*. 4X *dpp*; zen^- embryos were derived from the stock *CyO23, P[dpp$^+$]*/+; zen^{w36}*/TM3, hb-lacZ* (1/16 of the embryos). The double heterozygous embryos, dpp^{hr4}/+; zen^{w36}/+, were identified by the lack of *lacZ* staining in embryos derived from a cross of the dpp^{hr4} and zen^{w36} stocks (1/4 of the embryos).

Ectopic expression of *zen* and *zen-Del*

The *zen* cDNA (+10 to +1234 from the transcription starting site) (Rushlow et al., 1987a) was cloned into pUAST (Brand and Perrimon, 1993) via *Eco*RI and *Xba*I sites on the 5′ and 3′ ends, respectively. The *zen-Del* cDNA was made by PCR mutagenesis (Expand High Fidelity PCR system, Roche Applied Science) using oligos spanning the deletion region (amino acids 152-198) and cloned into pUAST via the *Eco*RI and *Xba*I sites. Transgenic flies were generated by the standard transformation protocol (Spradling and Rubin, 1982). Flies carrying UAS-*zen* and UAS-*zen-Del* were crossed to stripe-2 *eve-Gal4* drivers (gift from S. Small) and the expression of ectopic *zen* or *zen-Del* proteins was confirmed by staining with anti-Zen antibodies. Guinea pig or rabbit anti-Zen antibodies were generated (Covance) as described by Rushlow et al. (Rushlow et al., 1987b). To obtain uniform early embryonic expression of the UAS-*zen* and UAS-*zen-Del* transgenes, a maternal *Gal4* driver was used in which the *GAL4-VP16* fusion protein is expressed maternally under the control of the *α–tubulin 67C* promoter. These were further crossed into a zen^{w36}*/TM3, hb-lacZ* background for the *zen* mutant rescue experiments.

In vitro mutagenesis and transgenic analysis

The *Race* 533 bp enhancer DNA was kindly provided by M. Levine (Rusch and Levine, 1997). Deletions and point mutations were created by PCR mutagenesis. An internal deletion of 66 bp (nucleotides 432-497 of the *Race* enhancer) deletes most of the Mad-binding region. The two proximal Zen-binding sites were mutated as follows: ATATTAAT was changed to ATCTAGAT, and ATTAAAAATAAATAAT was changed to TAGAAAAATAACTGCA. *Race-lacZ* constructs were prepared by subcloning the wild-type and mutated versions of the *Race* enhancer into a modified Casper transformation vector that contains the minimal promoter sequence from the *even-skipped* gene fused to the *lacZ* reporter gene (*eve-lacZ* Casper) (Small et al., 1992). At least three transformant lines for each construct were tested.

In situ hybridization and antibody staining

Wild-type, mutant and transgenic embryos were fixed, hybridized with *zen*, *Race*, *hnt* or *lacZ* antisense RNA probes, and stained (Roche Molecular Biochemicals), dehydrated and mounted in araldite (Polysciences) as described by Rushlow et al. (Rushlow et al., 2001). Anti-PMad polyclonal antibodies were kindly provided by P. ten Dijke (Persson et. al., 1998) and used at a final dilution of 1:1000 in PBS. Secondary anti-rabbit antibody staining was performed using the Vectastain ABC kit (Vector Labs). Embryo

preparations were photographed using DIC optics on a Nikon FX-A microscope.

Bacterial expression of Zen, Zen-Del, Mad and Medea

The GST-Zen and GST-Zen-Del fusion constructs were cloned by introduction of *Eco*RI sites at the initial ATG and at the 15th nucleotide downstream of the stop TAA codon in the *zen* cDNA by PCR mutagenesis, followed by excision of the *Eco*RI fragment from the PCR product and ligation into the *Eco*RI site of the pGEX-4T-2 vector (Pharmacia). Expression plasmids encoding GST-Mad^{N} and GST-Medea fusion protein containing the N-terminal MH1 domains were obtained from A. Laughon (Kim et al., 1997) and M. Frasch (Xu et al., 1998), respectively. The expression and the purification of the recombinant proteins were carried out as described before (Kirov et al., 1993). The concentration of the isolated proteins was determined by SDS-PAGE after staining with Coomassie R-250, together with defined amounts of bovine serum albumin.

In vitro DNA binding assays

DNAse I footprint analyses were carried out as previously described (Kirov et al., 1993). The 533 bp *Race* enhancer was originally cloned into the pBluescript KS+ vector (Stratagene) *Not*I site (Rusch and Levine, 1997). Three fragments were generated for footprint analysis using the *Bss*HI and *Xho*I sites on each side of the vector polylinker: *Bss*HI-*Apo*I (–50 to 163), *Hin*fII-*Tth*III (107 to 349) and *Tth*III-*Xho*I (349-603). The electrophoretic mobility shift assays were performed as described before (Kirov et al., 1993) except that the electrophoresis was run at room temperature. The sequence of the 42 bp oligonucleotide (*Race* sequences 486-527) spanning the wild-type Smad-binding sites and the proximal Zen-binding site is (consensus core sites are underlined): 5′-TCAGACGCGACTAAGCCGATCTCGCATTAAAAATAAATAATG-3′. The Smad-binding site mutations are (mutated core sites are underlined): 5′-TTAGATGCGAGTAAGATGATCTCGCATTAAAAATAAATAATG-3′. The Zen-binding site mutations are (mutated core sites are underlined): 5′-TCAGACGCGACTAAGCCGATCTCTCTAGAAAAATAACTCGAG-3′. The sequence of the 128 bp DNA fragment is (consensus core sites are underlined; vector sequences are in lower case): 5′gaattcgcccttAACGTCGGCTTATCTTCGCGCCTACCTGGCCGAGAACCCCAGACGGATTGGAAACATCAGACGCGACTAAGCCGATCTCGCATTAAAAATAAATAATGCTCGAGaagggcgaattc-3′. The end-labeled oligonucleotides or fragment, which was isolated from a subclone of the *Race* enhancer by *Eco*RI digestion, were added to the reactions containing Zen, Mad, Medea or combinations of different proteins and incubated on ice for 30 minutes before loading on the gel.

Protein interaction assays

GST pull-down assays were carried out as previously described (Kirov et al., 1996). Wild-type (and mutant forms) of *zen* were cloned into the pAR vector (*Nde*I and *Eco*RI sites) (Rosenberg et al., 1987) by introduction of an *Nde*I site at the initial (or internal sites to make truncated proteins) and an *Eco*RI site four nucleotides downstream of the stop codon (or at internal sites to make truncated proteins). These constructs were expressed in vitro with the TNT Coupled Reticulocyte lysate system (Promega) using the T7 promoter in the pAR vector. Expression of these proteins was confirmed by electrophoresis on a 12% SDS polyacrylamide gel.

Results

zen encodes a homeodomain protein of the Antennapedia class, and is initially expressed broadly like *dpp* but becomes restricted to the dorsal most region, the presumptive amnioserosa (Doyle et al., 1986). This refinement of the *zen* pattern is dependent on Dpp, and proceeds simultaneously with the refining PMad gradient (Rushlow et al., 2001). Thus, by mid-late stage 5, *zen* transcripts are present only where there are peak levels of PMad, in the dorsal most cells. At this time, *Race* transcripts begin to accumulate in the same domain (Fig. 1A) (Tatei et al., 1995). *Race* activation is known to be dependent on *dpp* and *zen* as *Race* is not transcribed in either mutant (Tatei et al., 1995; Rusch and Levine, 1997). To better understand the mechanism underlying the requirement for both genes, we first examined *Race* expression in different genetic backgrounds that alter levels of *dpp* and *zen*, and then performed molecular analyses with Smad and Zen proteins to characterize protein-protein and protein-DNA interactions with the well-defined *Race* enhancer (Rusch and Levine, 1997; Wharton et al., 2004). We are particularly interested in how Dpp morphogenetic activity is interpreted by high level target genes so they come to be expressed only in the peak level domain: the presumptive amnioserosa.

dpp and *zen* are both required to activate *Race*

dpp^{hr4} is a weak *dpp* allele (Wharton et al., 1993) and *sog*^{SY06} is a strong *sog* allele (Ferguson and Anderson, 1992b). Mutant embryos of both genotypes fail to develop amnioserosa (Wharton et al., 1993; Zusman et al., 1988) presumably because of insufficient Smad signaling. In *dpp*^{hr4} and *sog*^{SY06}

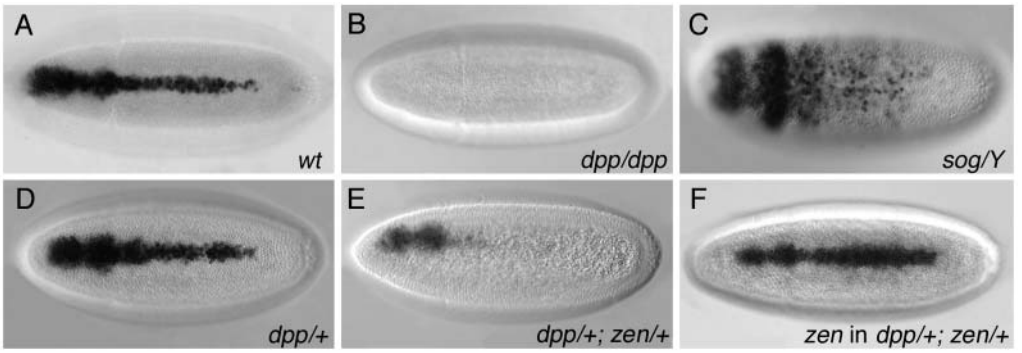

Fig. 1. *Race* expression depends on high levels of *dpp* and *zen* activity. Dorsal views of stage 5/6 embryos with anterior towards the left. Embryos were hybridized with *Race* (A-E) or *zen* (F) probes. (A) Wild type. *Race* transcripts are present in a five- to six-cell wide dorsal stripe in the main body region and in two broader patches in the region that will form head structures. (B) *Race* is absent in *dpp*^{hr4} homozygotes. (C) *Race* is expressed weakly in a broad domain in the main body region of *sog*^{SY06} hemizygous embryos. The head domains are relatively strong and also broader. (D) *Race* expression is normal in *dpp*^{hr4} heterozygotes. (E,F) *Race* transcripts are absent from the main body region, but appear normally in the head region of *dpp*^{hr4}/+; *zen*^{w36}/+ embryos (E), although *zen* expression is normal (F). The expression in the head domains appears to be less sensitive to a drop in *dpp* and *zen* activities.

mutants, PMad does not accumulate in the dorsalmost five or six cells, but instead fades quickly in dpp^{hr4}embryos and is present in a relatively strong broad domain in sog^{SY06} embryos (Rushlow et al., 2001). Correspondingly, the broad early *zen* pattern does not refine in either mutant, but follows closely PMad activity. The observed changes in the *zen* pattern reflects the failure of these embryos to generate a steep BMP gradient with the peak levels of PMad necessary for refined *zen* expression (Rushlow et al., 2001). *Race* transcripts are absent in dpp^{hr4} mutants (Fig. 1B). In sog^{SY06} mutants, *Race* expression is observed in a broad spotty pattern in the middle body region that fades quickly by gastrulation (Fig. 1C). These results taken together indicate that high levels of both PMad and Zen, above the levels present in each mutant, are required for proper *Race* activation.

dpp^{hr4}/+ heterozygous embryos have normal *Race* expression (Fig. 1D). Similarly, zen^{w36}/+ heterozygotes also have normal *Race* expression (data not shown). However, in double heterozygous dpp^{hr4}/+; zen^{w36}/+ embryos, *Race* expression is absent in the middle body region (Fig. 1E). *zen* expression appears normal in these embryos (Fig. 1F) as does PMad staining (data not shown). Thus, although the level of Dpp activity in these embryos is sufficient to drive refined *zen* expression, it is not able to compensate for the reduction in *zen* dose in order to properly activate *Race.* Likewise, there is not enough Zen to compensate for the reduction in Dpp activity. Again, we conclude that *Race* responds to high levels of combined Zen and PMad activities.

Race can be activated by low levels of PMad if Zen is present

To further investigate the combinatorial requirement for *dpp* and *zen*, we performed additional dosage studies. We first examined *Race* in embryos with four copies of *dpp* (4X *dpp*). The expression domains of peak-level PMad, *zen,* and *Race* are broader in these embryos covering about 12-14 cells (Fig. 2D-F). This demonstrates a clear correlation between peak levels of PMad/Zen and high-level Dpp target gene expression. Next, we examined *Race* expression in embryos with 4X *dpp* that lack *zen* activity. *Race* transcripts are present, but only in the dorsalmost five or six cells (Fig. 2I), comparable with that in 2X *dpp* embryos (Fig. 2C), even though peak levels of PMad and refined *zen* cover 12-14 cells in these embryos (Fig. 2G,H). Therefore, Dpp can activate *Race* in a *zen*-independent manner but only if expressed above wild-type levels, as was also concluded by Rusch and Levine (Rusch and Levine, 1997). However, *Race* is not activated across the entire domain of PMad, but only in the dorsal most region, suggesting that Dpp signaling is higher along the dorsal midline in a 4X *dpp* embryo

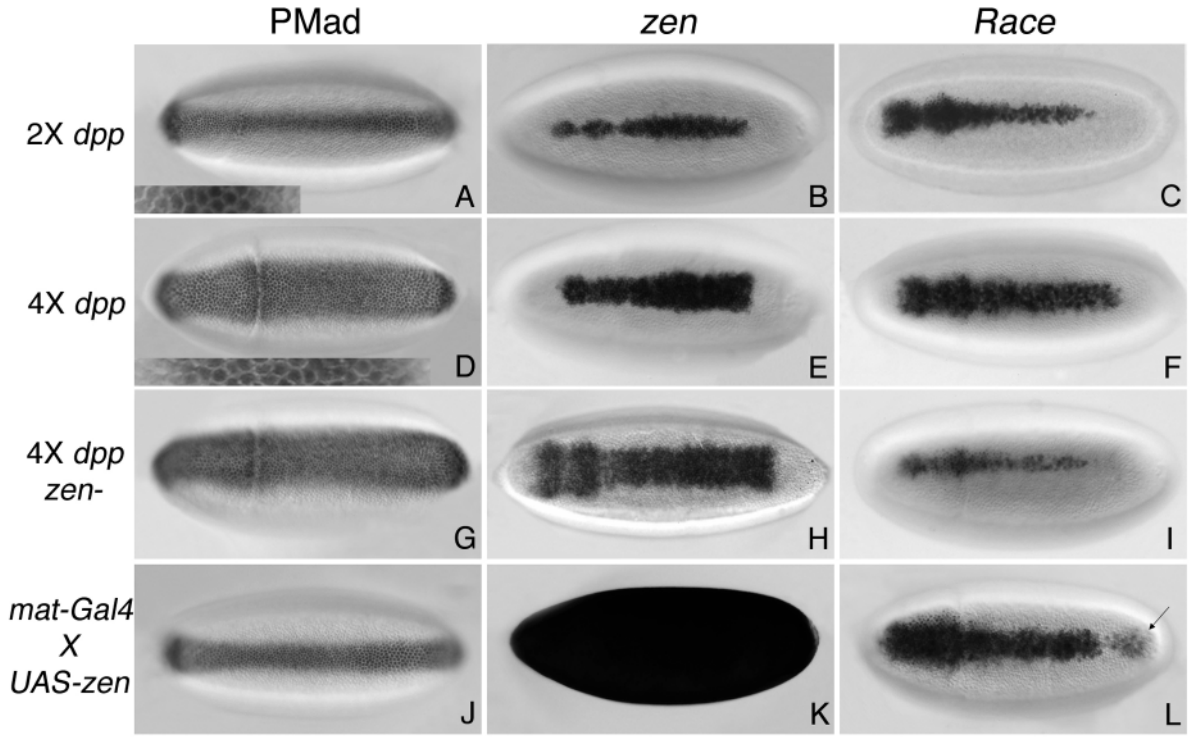

Fig. 2. The purpose of the BMP gradient peak is to set up the *zen* domain. All views of embryos are dorsal, except K (lateral). Embryos are of the genotypes wild type 2X *dpp* (A-C), 4X *dpp* (D-F), 4X *dpp*, zen^{w36} (G-I) and UAS-*zen* driven by maternal-*Gal4* (J-L; see Materials and methods), and were stained with anti-phospho Mad (PMad) antibodies (A,D,G,J), or hybridized with *zen* (B,E,H,K) or *Race* (C,F,I,L) RNA probes. Increasing the dose of *dpp* to four copies broadens the domain of peak level PMad to 10-12 cells (D; see inset for higher magnification view of one or two rows of cells, turned sideways). The domain of *zen* (E) and *Race* (F) broaden likewise. In *zen* mutant embryos with 4X *dpp*, the *zen* domain broadens (H), but that of *Race* does not (I), presumably because Zen proteins are absent (Rushlow et al., 1987). When *zen* is ubiquitously expressed throughout the embryo (K), the *Race* domain broadens to encompass the lower-level PMad domain (L), indicating that lower levels of PMad can activate *Race* if Zen is present. Ectopic *Race* expression is also observed in the posterior region (L, arrow) where PMad is present.

than in a wild type 2X *dpp* embryo, though not obvious in our antibody staining experiment.

Is the role of Zen to potentiate Dpp activity by reducing the threshold of Dpp required for *Race* activation, or are Zen and Dpp activities interchangeable, one being able to replace the other if the total amount of activity reaches the threshold? To discern these two possibilities, we examined whether *zen* can activate *Race* in the absence of PMad by overexpressing *zen* ubiquitously throughout the embryo using a maternal-*GAL4* driver and *UAS-zen* (Fig. 2K; see Materials and methods). The PMad staining pattern is normal in these embryos (Fig. 2J). However, the *Race* stripe is broader than normal, including both the high-level and low-level PMad domains (Fig. 2L), and is also observed in the posterior region (see arrow). *Race* is not detectable in more lateral regions where there is no detectable PMad. Therefore, *zen* cannot activate *Race* in the absence of PMad, even when overexpressed, suggesting that *Race* responds to a combination of activities provided by Zen and Dpp, but these activities are not exchangeable. Only when Zen was fused to a strong activation domain, VP16, did it become independent of Dpp in activating *Race* (Rusch and Levine, 1997).

These results reveal a dual role for Dpp in *Race* activation. Peak levels of BMP/Smads define the domain of *zen* in the dorsalmost cells. Next, BMP/Smads, together with Zen, activate downstream targets such as *Race*; however, this function does not require peak-level Smads as *Race* was activated in regions of low-level Smads when Zen was ectopically expressed (Fig. 2L). Thus, we propose that the role of the peak of the BMP gradient is to set the *zen* domain. Hence, Zen defines the expression borders of the downstream high level targets (see Discussion). We sought to investigate the molecular mechanism underlying the combinatorial requirement of Zen and Smads for the activation of *Race* transcription.

Mad facilitates the binding of Zen to the *Race* enhancer

We assayed for Mad and Medea binding to the *Race* enhancer, a 533 bp DNA fragment that lies 1.5 kb upstream of the *Race* transcription start site (Rusch and Levine, 1997). When fused with a *lacZ* reporter gene, this DNA fragment drives a similar expression pattern to that of wild-type *Race* mRNA (Fig. 3B) (Rusch and Levine, 1997). We performed DNA-binding assays with recombinant GST fused Mad protein and GST fused Medea protein, both of which contain the DNA-binding MH1 domain and the linker region (Kim et al., 1997; Xu et al., 1998).

DNase I footprinting assays revealed a Mad-binding region in the *Race* enhancer (Fig. 3A, lanes 3-5). It spans 82 bp (Fig. 3A, nucleotides 421-502 in blue and purple below the footprint) and is highly GC rich, containing several GNCN motifs (boxes labeled a-g on sequence), a configuration that has been shown to mediate strong inducible reporter gene responses upon binding of Smad

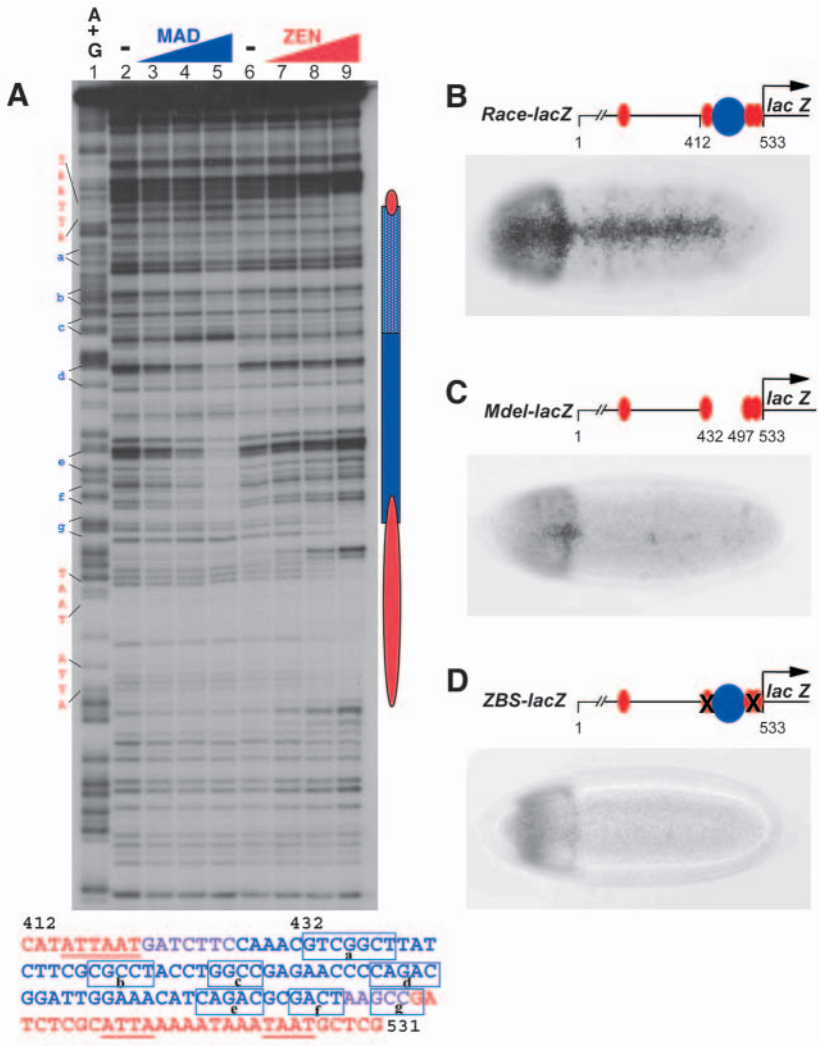

Fig. 3. The binding of both Mad and Zen to the *Race* enhancer is required for proper *Race* expression. (A) DNAse I footprinting analysis of Mad and Zen GST fusion proteins bound to a 255 bp fragment that includes the proximal region of the *Race* 533 bp enhancer (349-533) and 70 nucleotides from the Bluescript vector. The fragment is end-labeled at the vector end. Lane 1, chemical degradation of the probe on G+As; lanes 2 and 6, DNAse I digestion of the DNA probe. Increasing amounts of Mad (500 ng, 1500 ng and 4500 ng in lanes 3-5 respectively) and Zen (20 ng, 60 ng and 200 ng in lanes 7-9 respectively) were incubated with the fragment prior to DNAse I digestion. The region protected by Mad is depicted as a blue rectangle, the hatched half denoting weaker protection. The regions protected by Zen are shown as red ovals. The nucleotide sequence of the protected regions are shown below the gel with the overlap between the Zen and Mad footprints shown in purple. Putative core binding sites are underlined for Zen (Han et al., 1989) and boxed for Mad (boxes a,b,c,f,g) (Kim et al., 1996) and Medea (boxes d,e) (Xu et al., 1998; Pyrowolakis et al., 2004). The boxes are also marked on the G+A sequence. (B) Schematic representation of the full-length *Race* 533 bp enhancer fused to a *lacZ* reporter gene, and a transgenic embryo carrying this construct in situ hybridized with *lacZ* probes. *lacZ* expression is identical to the *Race* pattern. The ring of staining in the head region is an artifact of the vector. (C) Embryo carrying a deletion of the Mad-binding region (nucleotides 432-497). *lacZ* expression is severely reduced. (D) Embryo carrying mutations in the ATTA core sites of the Zen-binding sites (underlined, see Materials and methods). *lacZ* expression is absent.

complexes in cell culture (Johnson et al., 1999). The Mad-binding region contains a strongly protected area (nucleotides 464-502) (see also Wharton et al., 2004) and a weakly protected area (nucleotide 421-463) demarcated by solid and hatched rectangles, respectively (see Fig. 3 legend).

A 66 bp deletion of this region (nucleotides 432-497) abolishes the in vitro footprint binding of Mad (data not shown), and also greatly reduces in vivo expression of the *Race*-enhancer reporter gene, *Mdel-lacZ* (Fig. 3C), suggesting that Mad can directly activate *Race* via binding to this region. The observed residual activity of this reporter might be due to the remaining Smad-binding site (box g). We also detected four Zen-binding sites in the *Race* enhancer, three of which are shown in the footprint in Fig. 3A (lanes 6-9; nucleotides in red). Point mutations in all four of the ATTA core sites abolished *lacZ* reporter expression (Rusch and Levine, 1997), as did mutations in the three most proximal sites (Fig. 3D; *ZBS-lacZ*).

The above data favor the existence of a relatively short regulatory module containing a cluster of Smad-binding sites bordered by Zen-binding sites that regulates the essential aspects of early *Race* expression. Similarly, short sequences containing multiple Smad sites and other transcription factor binding sites have been found to regulate the Dpp targets *tin* (Xu et al., 1998) and *Ubx* (Saller and Bienz, 2001) in *Drosophila*.

The closely apposed Smad- and Zen-binding sites in the *Race* regulatory module hinted at the possibility that their combined activity, which is essential for *Race* activation, might partially depend on their direct interaction. The most immediate result from such an interaction could be to facilitate the binding of one or the other protein to DNA. To test this, we tried to detect cooperative binding of Mad and Zen proteins to DNA. In preliminary experiments, we found the range of concentrations of Zen and Smad proteins that produce complexes with a 42 bp oligonucleotide spanning a cluster of three Smad-binding sites and the two most proximal Zen sites. Then by incubating one component with suboptimal amounts of the other, which by itself is not sufficient to produce complexes, we expected that the DNA binding of the protein of suboptimal concentration would increase if there is cooperative DNA binding, and/or possibly form supershift complexes containing both proteins.

Mad and Medea form similar complexes with the 42 bp oligonucleotide (Fig. 4B, lanes 2-3), and do not bind oligonucleotides in which the three Smad sites (CAGAC, GACT, GCCG) were mutated (TAGAT, GAGT, GATG respectively; lanes 6-7). In experiments with the larger 122 bp fragment that contains seven Smad sites (Fig. 4A), mutation of the two CAGAC sites (boxes d and e in Fig. 3A and Fig. 4A) prevented Medea binding (data not shown), indicating that Medea binds the CAGAC site, as was recently shown by Pyrowolakis et al. (Pyrowolakis et al., 2004). Zen forms two complexes with the 42 bp oligonucleotide that have different mobilities from the Smad complexes (Fig. 4B, lane 4), and does not bind to oligonucleotides in which the core sites (ATTA, TAAT) were mutated (TAGA, TCGA, respectively; Fig. 4B, lane 12). When a concentration of Mad that does not produce detectable Mad complexes was incubated with varying Zen protein concentrations and the122 bp DNA fragment, the intensity of the Zen complexes increased compared with when Mad was not added to the reactions (Fig. 4C, lanes 10-12

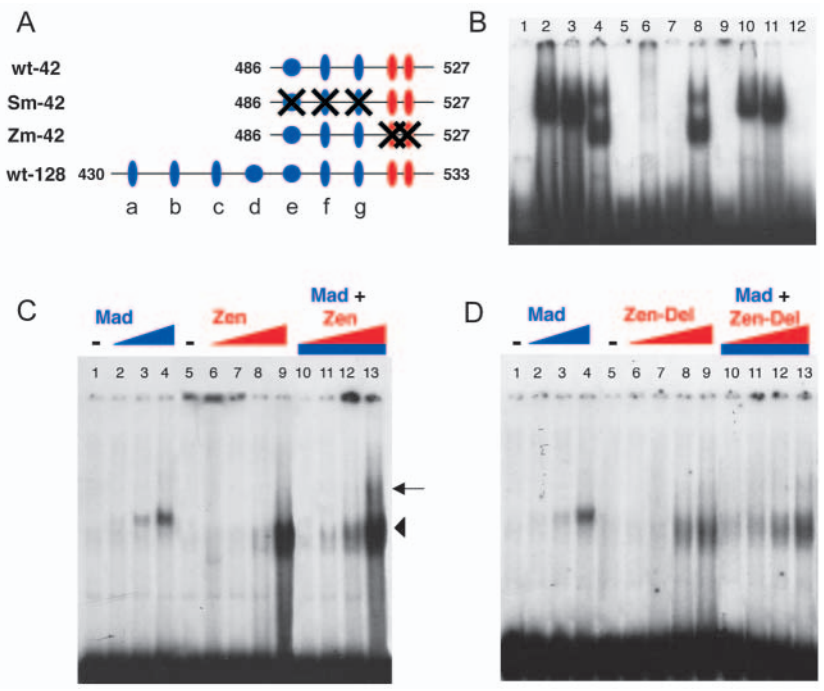

Fig. 4. Zen binding is enhanced in the presence of Mad. (A) Schematic representation of the 42 bp wild-type (wt-42) and mutant oligonucleotides that eliminate the core Smad- (Sm-42) or Zen- (Zm-42) binding sites, and the 128 bp DNA fragment from the *Race* enhancer used in electrophoretic mobility shift assays (EMSAs) showing Medea- (blue circle), Mad- (blue ovals) and Zen- (red ovals) binding sites. (B) DNA binding requires the core consensus sites. ^{32}P-labeled wild type (lanes 1-4) and mutant oligonucleotides (Sm-42, lanes 5-8; Zm-42, lanes 9-12) were incubated with no protein (lanes 1,5,9); and 100 ng Mad (lanes 2,6,10), 100 ng Medea (lanes 3,7,11) or 10 ng Zen (lanes 4,8,12). Mad and Medea produce a single complex of bound probe, whereas Zen produces two complexes. The slower migrating Zen complex could be due to a Zen/Zen/DNA complex. Mutant Sm-42 (or Zm-42) eliminated the binding of Mad/Medea (or Zen) without affecting the binding of Zen (or Mad/Medea). (C) ^{32}P-labeled wild-type DNA fragments were incubated with no protein (lanes 1 and 5), increasing amounts of Mad (1 ng, 2 ng, and 5 ng in lanes 2-4, respectively), increasing amounts of Zen (0.1 ng, 0.3 ng, and 1 ng in lanes 6-9, respectively) or increasing amounts of Zen in the presence of 1 ng of Mad (lanes 10-13). The amounts of Zen in lanes 10-13 were the same as in lanes 6-9. More Zen complexes are shifted in lanes 10-13 compared with lanes 6-9. In lane 13, a supershifted complex (arrow) is visible above the Zen complex (arrowhead) that may be due to the formation of a Mad/Zen/DNA complex or a Zen/Zen/DNA complex. (D) Similar experiment as in C, except that Zen-Del was used instead of wild-type Zen. The same amounts of proteins were used as in C. Enhancement of Zen binding by Mad was not observed.

compared to lanes 6-8). Complexes that could be interpreted as Mad/Zen supershifts were not clearly visible as distinct complexes. Similar experiments using the DNA fragments with mutated Smad sites showed little if any enhancement of Zen binding by small amounts of Mad (data not shown), indicating that the observed cooperative binding depends on Mad interaction with DNA.

In the reverse experiment, incubating Zen protein at a concentration that does not produce detectable complexes with varying amounts of Mad, neither the formation of a new complex nor any increase in Mad complexes was observed (data not shown). We did, however, observe an enhancement of Zen binding in the lanes with low Zen and increasing Mad concentrations (data not shown), consistent with the results from the previous experiment.

Mad-Zen protein interaction is necessary for *Race* activation

To test for direct physical protein-protein interactions between Zen and Mad proteins, we performed GST pull-down assays with GST-Mad^N and in vitro translated full-length and truncated Zen proteins, (Fig. 5A,B). Full-length Zen clearly interacts with Mad (Fig. 5C, lanes 1-2). Our results confirm the recent report of the Mad-Zen interaction found in a genome-wide yeast two-hybrid screen (Giot et al., 2003). By testing a series of truncated Zen proteins (Fig. 5C, lanes 3-14), we mapped the domain of the Zen protein involved in the interaction with Mad to be within the 48 amino acids C-terminal to the homeodomain (amino acids 152-199). Removal of this region by an internal deletion (Fig. 5A, Zen-Del) also abolishes the ability of Zen to interact with GST-Mad^N (Fig. 5C, lanes 15-16). Though the recombinant Zen protein with this deletion, Zen-Del, binds DNA similarly to full-length Zen (Fig. 4D, lanes 6-9), the cooperative binding to the wild-type DNA fragment containing Mad- and Zen-binding sites is not observed (Fig. 4D, lanes 10-13).

To test the functional relevance of the Mad-Zen physical interaction, we ubiquitously expressed the Zen-Del protein in the early embryo. As mentioned before, *UAS-zen* driven by maternal-*Gal4* can ectopically activate *Race* in the posterior region of the dorsal midline, as well as more laterally along the DV axis (Fig. 6A). By contrast, the deletion construct *UAS-zen-Del* fails to elicit any ectopic *Race* expression (Fig. 6B). Indeed, *Race* expression in maternal-*Gal4/UAS-zen-Del* embryos is indistinguishable from that in wild-type embryos, demonstrating that the Mad-Zen interaction is required for ectopic *Race* expression.

As a second test for functional relevance of the Mad-Zen interaction, we examined whether the loss of *Race* expression in *zen* mutants could be rescued by ubiquitously expressed Zen, and if so, was the Mad interaction domain required. Wild-type *UAS-zen* driven by the maternal-*Gal4* driver was able to restore *Race* expression in *zen^{w36}* null mutants (Fig. 6C). By contrast, *UAS-zen-Del* failed to rescue *Race* expression (Fig. 6D). This result suggests that the Mad-Zen protein interaction is necessary for endogenous *Race* activation. In summary, both in vitro and in vivo evidence suggests that a physical interaction between Mad and Zen is essential to the underlying mechanism involved in *Race* activation.

Discussion

We have studied the role of the BMP morphogen in regulating high level target genes in the early *Drosophila* embryo. Strikingly, our results present evidence that the primary role, and possibly the only role, of the peak level of the BMP gradient is to activate *zen*. Once *zen* expression becomes restricted to the presumptive amnioserosa in the cellular blastoderm embryo, Zen protein and the BMP signal-transducing Smads act in concert to activate the high level BMP target gene *Race*. We have shown that Zen and Smads bind directly to adjacent sites in a short regulatory module in the *Race* enhancer, and this interaction with DNA is essential for *Race* activation. We also found that *Race* activation depends on direct protein-protein interaction between Mad and Zen. Our results showing enhanced DNA binding of Zen to the *Race* enhancer in the presence of Mad provide a further explanation for this interaction, to ensure the synergistic action of Zen and Smads necessary to activate high level target genes in the presumptive amnioserosa.

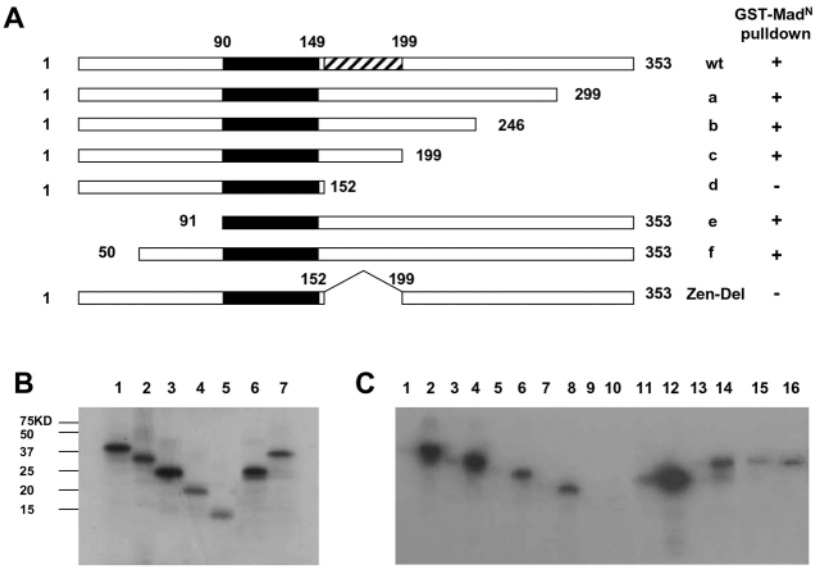

Fig. 5. Mad and Zen proteins interact. (A) Schematic representation of the full-length and truncated forms of Zen proteins used in the in vitro protein interaction assays. Black bars represent the homeodomain of Zen (amino acids 90-149) (Rushlow et al., 1987) and the hatched bar conatins the putative Mad-Zen interaction domain (amino acids 152-199). (B) Autoradiogram of 5% of the amounts of ^{35}S-labeled in vitro translated Zen proteins used in each binding reaction. Lane 1, full-length Zen; lanes 2-7, truncated Zen proteins a-f. (C) Results of GST pull-down assays with full-length and truncated Zen proteins. Odd-numbered lanes were reactions using GST protein (negative controls). Even-numbered lanes were reactions using GST-Mad^N with the following Zen proteins: lane 2, full length; lane 4, a; lane 6, b; lane 8, c; lane 10, d; lane 12, e; lane 14, f; lane 16, Zen-Del. Results are also summarized on the right side of A. Zen-Del has background binding with GST alone. In repeated experiments, the difference between GST and GST-Mad^N lanes for Zen-Del was somewhat variable and consistently insignificant.

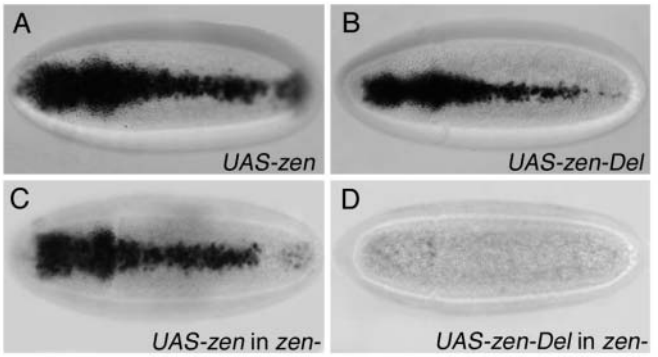

Fig. 6. The Mad-Zen interaction is necessary for *Race* activation. Late stage 5 embryos were hybridized in situ with a *Race* probe (A,B) or *Race* and *lacZ* probes (to determine the *zen* mutant embryos; C,D). (A) Embryo expressing UAS-*zen* driven by the maternal *Gal4* driver. Ectopic *Race* expression is visible in the posterior region and the dorsal stripe is broader. (B) Embryo expressing UAS-*zen*-Del driven by the maternal *Gal4* driver. Ectopic *Race* transcripts are absent. (C) zen^{w36} mutant embryo expressing UAS-*zen* driven by the maternal *Gal4* driver. *Race* expression is restored. (D) zen^{w36} mutant embryo expressing UAS-*zen*-Del driven by the maternal *Gal4* driver. *Race* expression is absent, indicating that Zen-Del is not able to rescue the *zen* mutant phenotype.

Race is activated by a combinatorial mechanism

Specific activation or repression of transcription by a combination of transcription factors is a common theme in the regulation of developmentally important genes (Howard and Davidson, 2004). The results from our genetic analysis and the molecular dissection of the *Race* enhancer clearly show that *Race* is activated by the combined action of Smads and Zen. Although Smads can single handedly activate *Race* when overexpressed (Fig. 2I), under normal circumstances concurrent Zen activity is required. Why are both Smads and Zen necessary?

Zen may act to restrict target gene expression specifically to the presumptive amnioserosa. As the Dpp pathway is used repeatedly during development, other factors must function in combination with Dpp to ensure tissue specificity (see Affolter and Mann, 2001; Reim et al., 2003). The ectopic expression studies support this idea. In normal embryos, *Race* is activated only in regions where there are peak levels of PMad and Zen. In embryos where Zen is ubiquitously expressed, *Race* can now be activated in regions where there are lower levels of PMad (Fig. 2L), indicating that high level PMad is not the determining factor for amnioserosa tissue specificity. Rather PMad allows expression, and Zen determines the border of expression. We interpret the overexpression studies where Dpp can activate *Race* alone (Fig. 2I) (Rusch and Levine, 1997) to be situations where there are such high levels of Smads that *Race* and *hnt* become activated promiscuously, and hence differential regulation is lost. In normal embryos, the combination of Smads and Zen ensures that the high level target genes are activated only in the presumptive amnioserosa.

On the other hand, why the need for Smads? One role for Smads is suggested from the observation that Smads facilitate the binding of Zen to the *Race* enhancer (Fig. 4). It is well established that Hox proteins often require co-factors for DNA binding to target enhancers (reviewed by Mann and Affolter, 1998). For example, composite sites that also bind the co-factor Extradenticle (Exd) ensure a greater selectivity for binding over the higher frequency Hox core site such as TAAT. In other examples, binding sites for signaling pathway effectors lie close to Hox/Selector-binding sites (see Guss et al., 2001; Affolter and Mann, 2001). The closely apposed Zen- and Smad-binding sites in the *Race* enhancer is one such scenario, as Zen can be thought of as a Selector gene (Rushlow and Levine, 1990). Our studies add to this idea of Smads and Selector cooperativity by demonstrating enhanced binding of Zen in the presence of Smads. Though the enhancement we observe in our in vitro assays is not dramatic, it is possible that in the embryo a moderate enhancement is functionally significant as is the twofold doubling of the *dpp* dose.

Another potential role of Smads was suggested from previous overexpression studies (Rusch and Levine, 1997). Zen was only able to activate *Race* in the absence of Dpp if fused to a strong activation domain derived from VP16. This suggests that Smads provide a transactivation function different from that of Zen. The Smad MH2 domain has been shown to interact with the transcriptional co-activators CBP and p300 (Waltzer and Bienz, 1999; Shi and Massagué, 2003). Zen has not yet been analyzed for interaction with transcriptional co-activators, however, the activation domain of Zen lies within the C-terminal 119 amino acids (Han et al., 1989), and does not overlap with the homeodomain or the Mad interaction domain (Fig. 5). Mechanistically, the difference in the activation potential between Zen and Smads could be due to their ability to recruit different co-activators to the transcriptional machinery.

Mechanisms of threshold responses to the BMP morphogen gradient

Gradients of morphogens provide positional information to the cells by activating different genes at different threshold concentrations. In early *Drosophila* embryos, the transcriptional threshold responses to the Bicoid (Driever et al., 1989) and Dl (reviewed by Stathopoulos and Levine, 2002) morphogens have been extensively studied. The major mechanisms by which thresholds are established exploits the DNA-binding affinities of Bcd and Dl to their operator sites, as well as synergistic interactions with other transcription factors bound to the *cis*-regulatory sequences.

The BMP morphogen gradient also elicits different threshold responses from its targets, and, as discussed above, a combinatorial mechanism is used to activate *Race*, a high level Dpp target. Our genetic results indicate that *Race* (Fig. 1), and also another high level target *hnt* (M.X., unpublished), are activated only when a specific threshold of Zen and Smad activities are reached. In *sog* mutant embryos, Zen and Smad concentrations are relatively high, though below peak levels (Rushlow et al., 2001), and there is just enough of their combined activity to weakly activate *Race* (Fig. 1C) and *hnt* (data not shown). By contrast, in the double heterozygous embryos dpp^{hr4}/+; zen^{w36}/+, *Race* is not activated (Fig. 1E) because Zen and Smad concentrations are below the threshold levels required for activation.

A simple way to explain these results is if the *Race* enhancer has low affinity to Zen and Smad proteins in vivo. To transcribe *Race* effectively would then require relatively high concentrations of the proteins, which are indeed reached in the

dorsalmost cells. It has been known for some time that the enhancers of the high level Dl targets contain binding sites with lower affinity for Dl compared with genes responding to lower levels of Dl (reviewed by Stathopoulos and Levine, 2002). Recently, it has been shown that increasing the affinities of Smad-binding sites in the *Race* enhancer broadens the *Race* expression domain, which argues that the affinities of the Smad-binding sites in this high level Dpp target gene enhancer are low (Wharton et al., 2004). Our results suggest that cooperative binding between Smads and Zen, which is dependent on their physical interaction, should increase their binding to the *Race* enhancer (Figs 4-6). It is possible that interacting with Smads at the protein level either increases the binding affinity of Zen or effectively increases the local concentration of Zen when Smads bind the adjacent sites. This in turn leads to a robust transcriptional response of *Race*. The overexpression results are consistent with such a model. Ectopic Zen can only activate *Race* if some detectable level of PMad is present (Fig. 2L), and in addition Zen must contain the Smad interaction domain (Fig. 6B).

How are the lower level target genes activated? *ush* and *rho* are expressed in a broader domain, the border coinciding exactly with that of low level PMad staining. The Zen domains, however, do not; refined *zen* is not broad enough, while early Zen is too broad encompassing the entire dorsal domain, though Zen could possibly be graded in this region (Rushlow et al., 1987b). Thus, it is possible that this class of target genes relies on a mechanism that uses numerous high-affinity Smad sites, and/or synergistic action of Smads with other co-factor(s) besides Zen. Such a mechanism resembles the activation of target genes in the neurogenic ectoderm of the embryo by Dl (reviewed by Stathopoulos and Levine, 2002). It has been shown that the threshold responses from these genes depend on high-affinity Dl-binding sites, as well as synergistic interactions of Dl with bHLH transcription factors (Ip et al., 1992a; Jiang and Levine, 1993).

The *pnr* expression domain, which is about three times broader than *ush*, may represent a third threshold of Dpp activity. However, *pnr* is a different type of target gene compared with the prior classes in that it is repressed by Brk, which is present in a reverse gradient to Dpp (Jazwinska et al., 1999a; Ashe et al., 2000; Müller et al., 2003). In *brk* mutants, *pnr* expands into the ventral region, while *Race* and *ush*, for example, are unchanged. We expect that the *pnr* gene enhancer contains Brk-binding sites, whereas we observed in this study that the *Race* enhancer does not (M.X., unpublished). Brk binding sites often overlap with GNCN sites (Sivasankaran et al., 2000; Rushlow et al., 2001; Zhang et al., 2001), and it is possible that in the embryo, as in the wing disc, a concentration-dependent competition between Smads and Brk establishes the expression domains of the target genes regulated by both inputs (Rushlow et al., 2001). However, whether direct competition for binding can generate threshold responses remains to be seen. In summary, it appears that different classes of Dpp target genes are regulated by different combinations of transcription factors.

Race activation by Dpp and Zen resembles a feed-forward loop

One of the simple regulatory motifs used in transcriptional networks is the feed-forward (Lee et al., 2002) or self-enabling (Kang et al., 2003) mechanism, whereby one regulator controls a second regulator and then both bind a common target gene. It has been shown both in prokaryotes (Shen-Orr et al., 2002) and yeasts (Lee et al., 2002) that this mode of regulation appears relatively frequently and is favored over others, e.g. autoregulation motifs, single input motifs in which one regulator controls several genes, or regulator chain motifs whereby one gene regulates a second which regulates a third, and so on. Such an over-representation of the feed-forward motif is probably due to its potential to provide enhanced sensitivity and temporal control to the transcriptional response. The feed-forward loop is especially suitable for eliciting precise threshold responses of morphogen targets as it allows a strong response of the target gene to small changes in the activity of the regulator that initiates the loop (Dpp), because of the combined action with the second regulator (Zen). In fact Bcd and Dl use mechanisms that are reminiscent of the feed-forward loop to activate their high level targets. Bcd regulates zygotic *hunchback* (*hb*) and together Bcd and Hb activate the downstream target *even-skipped* (*eve*) stripe 2 (Small et al., 1992), and Dl activates *sna* with the help of Twi (Ip et al., 1992b). It is striking that the three morphogen gradients involved in specifying the *Drosophila* embryonic axes use the feed-forward strategy to regulate downstream target genes.

The primary role of the BMP gradient peak is to set up Zen

An unexpected implication from our results concerns the role of the high end of BMP morphogen gradient. In *Drosophila* embryos, the refined *zen* domain depends on peak levels of BMP activity, and we have shown that Zen can activate high level targets as long as there is some level of PMad present to facilitate DNA binding. It can be then concluded that, for the high level targets, the role of Dpp is twofold: to set the domain of *zen*, which we can refer to as a primary target gene; and then to act in combination with Zen to activate the other, secondary, target genes such as *Race* and *hnt*. In addition, with respect to the BMP gradient in the *Drosophila* embryo, we further propose that the sole purpose of the peak of the gradient is to set up the *zen* domain.

The authors thank Mike Levine for the *Race* enhancer DNA and Peter ten Dijke for the anti-phospho-Smad 1 antibodies. We are indebted to Michael Klein, Yelena Shusterman and Nzovu Ulenga for help in making and analyzing transgenic embryos. This work was supported by a grant from the National Institutes of Health (GM63024). This investigation was conducted in a facility constructed with support from Research Facilities Improvement Grant C06 RR-15518-01 from the National Center for Research Resources, National Institutes of Health.

References

Affolter, M. and Mann, R. (2001). Legs, eyes, or wings: selectors and signals make the difference. *Science* **292**, 1080-1081.

Arora, K., Levine, M. and O'Connor, M. B. (1994). The screw gene encodes a ubiquitously expressed member of the TGF-β family required for specification of dorsal cell fates in the *Drosophila* embryo. *Genes Dev.* **8**, 2588-2601.

Ashe, H. L., Mannervik, M. and Levine, M. (2000). Dpp signaling thresholds in the dorsal ectoderm of the *Drosophila* embryo. *Development* **127**, 3305-3312.

Biehs, B., François, V. and Bier, E. (1996). The *Drosophila* short gastrulation

gene prevents Dpp from autoactivating and suppressing neurogenesis in the neuroectoderm. *Genes Dev.* **10**, 2922-2934.

Bier, E., Jan, L. Y. and Jan, Y. N. (1990). rhomboid, a gene required for dorsoventral axis establishment and peripheral nervous system development in *Drosophila melanogaster*. *Genes Dev.* **4**, 190-203.

Brand, A. and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401-415.

Campbell, G. and Tomlinson, A. (1999). Transducing the Dpp morphogen gradient in the wing of *Drosophila*: regulation of Dpp target genes by brinker. *Cell* **96**, 553-562.

Cubadda, Y., Heitzler, P., Ray, R. P., Bourouis, M., Ramain, P., Gelbart, W., Simpson, P. and Haenlin, M. (1997). u-shaped encodes a zinc finger protein that regulates the proneural genes achaete and scute during the formation of bristles in *Drosophila*. *Genes Dev.* **11**, 3083-3095.

Doyle, H. J., Harding, K., Hoey, T. and Levine, M. (1986). Transcripts encoded by a homeobox gene are restricted to dorsal tissues of *Drosophila* embryos. *Nature* **323**, 76-79.

Driever, W., Thoma, G. and Nusslein-Volhard, C. (1989). Determination of spatial domains of zygotic gene expression in the *Drosophila* embryo by the affinity of binding sites for the bicoid morphogen. *Nature* **340**, 363-367.

Ferguson, E. L. and Anderson, K. V. (1992a). Decapentaplegic acts as a morphogen to organize dorsal-ventral pattern in the *Drosophila* embryo. *Cell* **71**, 451-461.

Ferguson, E. L. and Anderson, K. V. (1992b). Localized enhancement and repression of the activity of the TGF-β family member, decapentaplegic, is necessary for dorsal-ventral pattern formation in the Drosophila embryo. *Development* **114**, 583-597.

Giot, L., Bader, J. S., Brouwer, C., Chaudhuri, A., Kuang, B., Li, Y., Hao, Y. L., Ooi, C. E., Godwin, B., Vitols, E. et al. (2003). A protein interaction map of *Drosophila melanogaster*. *Science* **302**, 1727-1736.

Guss, K. A., Nelson, C. E., Hudson, A., Kraus, M. E. and Carroll, S. B. (2001). Control of a genetic regulatory network by a selector gene. *Science* **292**, 1164-1167.

Han, K., Levine, M. S. and Manley, J. L. (1989). Synergistic activation and repression of transcription by *Drosophila* homeobox proteins. *Cell* **56**, 573-583.

Holley, S. A., Jackson, P. D., Sasai, Y., Lu, B., de Robertis, E. M., Hoffmann, F. M. and Ferguson, E. L. (1995). A conserved system for dorsal-ventral patterning in insects and vertebrates involving sog and chordin. *Nature* **376**, 249-253.

Howard, M. L. and Davidson, E. H. (2004). cis-Regulatory control circuits in development. *Dev. Biol.* **271**, 109-118.

Ip, Y. T., Park, R. E., Kosman, D., Bier, E. and Levine, M. (1992a). The *dorsal* gradient morphogen regulates stripes of *rhomboid* expression in the presumptive neuroectoderm of the *Drosophila* embryo. *Genes Dev.* **6**, 1728-1739.

Ip, Y. T., Park, R. E., Kosman, D., Yazdanbakhsh, K. and Levine, M. (1992b). dorsal-twist interactions establish *snail* expression in the presumptive mesoderm of the *Drosophila embryo*. *Genes Dev.* **6**, 1518-1530.

Jazwinska, A., Kirov, N., Wieschaus, E., Roth, S. and Rushlow, C. (1999a). The *Drosophila* gene brinker reveals a novel mechanism of Dpp target gene regulation. *Cell* **96**, 563-573.

Jazwinska, A., Rushlow, C. and Roth, S. (1999b). The role of brinker in mediating the graded response to Dpp in early *Drosophila* embryos. *Development* **126**, 3323-3334.

Jiang, J. and Levine, M. (1993). Binding affinities and cooperative interactions with bHLH activators delimit threshold responses to the dorsal gradient morphogen. *Cell* **72**, 741-752.

Johnson, K., Kirkpatrick, H., Comer, A., Hoffmann, F. M. and Laughon, A. (1999). Interaction of Smad complexes with tripartite DNA-binding sites. *J. Biol. Chem.* **274**, 20709-20716.

Kang, Y., Chen, C. R. and Massague, J. (2003). A self-enabling TGF-β response coupled to stress signaling: Smad engages stress response factor ATF3 for Id1 repression in epithelial cells. *Mol. Cell* **11**, 915-926.

Kim, J., Johnson, K., Chen, H., Carroll, S. and Laughon, A. (1997). *Drosophila* Mad binds to DNA and directly mediates activation of vestigial by Decapentaplegic. *Nature* **388**, 304-308.

Kirkpatrick, H., Johnson, K. and Laughon, A. (2001). Repression of Dpp targets by binding of brinker to Mad sites. *J. Biol. Chem.* **276**, 18216-18222.

Kirov, N., Zhelnin, L., Shah, J. and Rushlow, C. (1993). Conversion of a silencer into an enhancer: evidence for a co-repressor in dorsal-mediated repression in *Drosophila*. *EMBO J.* **12**, 3193-3199.

Kirov, N., Lieberman, P. M. and Rushlow, C. (1996). The transcriptional corepressor DSP1 inhibits activated transcription by disrupting TFIIA-TBP complex formation. *EMBO J.* **15**, 7079-7087.

Lee, T. I., Rinaldi, N. J., Robert, F., Odom, D. T., Bar-Joseph, Z., Gerber, G. K., Hannett, N. M., Harbison, C. T., Thompson, C. M., Simon, I. et al. (2002). Transcriptional regulatory networks in *Saccharomyces cerevisiae*. *Science* **298**, 799-804.

Mann, R. S. and Affolter, M. (1998). Hox proteins meet more partners. *Curr. Opin. Genet. Dev.* **8**, 423-429.

Mason, E. D., Konrad, K. D., Webb, C. D. and Marsh, L. J. (1994). Dorsal midline fate in *Drosophila* embryos requires *twisted gastrulation*, a gene encoding a secreted protein related to human connective tissue growth factor. *Genes Dev.* **8**, 1489-1501.

Minami, M., Kinoshita, N., Kamoshida, Y., Tanimoto, H. and Tabata, T. (1999). brinker is a target of Dpp in *Drosophila* that negatively regulates Dpp-dependent genes. *Nature* **398**, 242-246.

Müller, B., Hartmann, B., Pyrowolakis, G., Affolter, M. and Basler, K. (2003). Conversion of an extracellular Dpp/BMP morphogen gradient into an inverse transcriptional gradient. *Cell* **113**, 221-233.

Persson, U., Izumi, H., Souchelnytskyi, S., Itoh, S., Grimsby, S., Engstrom, U., Heldin, C. H., Funa, K. and ten Dijke, P. (1998). The L45 loop in type I receptors for TGF-β family members is a critical determinant in specifying Smad isoform activation. *FEBS Lett.* **434**, 83-87.

Pyrowolakis, G., Hartmann, B., Müller, B., Basler, K. and Affolter, M. (2004). A Simple Molecular Complex Mediates Widespread BMP-Induced Repression during *Drosophila* Development. *Dev. Cell*. **7**, 229-240.

Raftery, L. A. and Sutherland, D. J. (2003). Gradients and thresholds: BMP response gradients unveiled in *Drosophila* embryos. *Trends Genet.* **19**, 701-708.

Ramain, P., Heitzler, P., Haenlin, M. and Simpson, P. (1993). pannier, a negative regulator of achaete and scute in *Drosophila*, encodes a zinc finger protein with homology to the vertebrate transcription factor GATA-1. *Development* **119**, 1277-1291.

Reim, I., Lee, H. H. and Frasch, M. (2003). The T-box-encoding Dorsocross genes function in amnioserosa development and the patterning of the dorsolateral germ band downstream of Dpp. *Development* **130**, 3187-3204.

Rosenberg, A. H., Lade, B. N., Chui, D. S., Lin, S. W., Dunn, J. J. and Studier, F. W. (1987). Vectors for selective expression of cloned DNAs by T7 RNA polymerase. *Gene* **56**, 125-135.

Ross, J. J., Shimmi, O., Vilmos, P., Petryk, A., Kim, H., Gaudenz, K., Hermanson, S., Ekker, S. C., O'Connor, M. B. and Marsh, J. L. (2001). Twisted gastrulation is a conserved extracellular BMP antagonist. *Nature* **410**, 423-424.

Rusch, J. and Levine, M. (1997). Regulation of a dpp target gene in the *Drosophila* embryo. *Development* **124**, 303-311.

Rushlow, C. and Levine, M. (1990). Role of the zerknüllt gene in dorsal-ventral pattern formation in *Drosophila*. *Adv. Genet.* **27**, 277-307.

Rushlow, C., Doyle, H., Hoey, T. and Levine, M. (1987a). Molecular characterization of the zerknüllt region of the Antennapedia gene complex in *Drosophila*. *Genes Dev.* **1**, 1268-1279.

Rushlow, C. A., Frasch, M., Doyle, H. and Levine, M. (1987b). Maternal regulation of zerknüllt: A homeobox gene controlling differentiation of dorsal tissues in *Drosophila*. *Nature* **330**, 583-586.

Rushlow, C., Colosimo, P. F., Lin, M. C., Xu, M. and Kirov, N. (2001). Transcriptional regulation of the *Drosophila* gene zen by competing Smad and Brinker inputs. *Genes Dev.* **15**, 340-351.

Saller, E. and Bienz, M. (2001). Direct competition between Brinker and Drosophila Mad in Dpp target gene transcription. *EMBO Rep.* **2**, 298-305.

Saller, E., Kelley, A. and Bienz, M. (2002). The transcriptional repressor Brinker antagonizes Wingless signaling. *Genes Dev.* **16**, 1828-1838.

Shen-Orr, S. S., Milo, R., Mangan, S. and Alon, U. (2002). Network motifs in the transcriptional regulation network of *Escherichia coli*. *Nat Genet.* **31**, 64-68.

Shi, Y. and Massagué, J. (2003). Mechanisms of TGF-β Signaling from cell membrane to the nucleus. *Cell* **113**, 685-700.

Shimell, M. J., Ferguson, E. L., Childs, S. R. and O'Connor, M. B. (1991). The *Drosophila* dorsal-ventral patterning gene *tolloid* is related to human bone morphogenetic protein 1. *Cell* **67**, 469-481.

Shimmi, O. and O'Connor, M. B. (2003). Physical properties of Tld, Sog, Tsg and Dpp protein interactions are predicted to help create a sharp boundary in BMP signals during dorsoventral patterning of the Drosophila embryo. *Development* **130**, 4673-4682.

Sivasankaran, R., Vigano, M. A., Muller, B., Affolter, M. and Basler, K. (2000). Direct transcriptional control of the Dpp target omb by the DNA binding protein Brinker. *EMBO J.* **19**, 6162-6172.

Small, S., Blair, A. and Levine, M. (1992). Regulation of even-skipped stripe 2 in the Drosophila embryo. *EMBO J.* **11**, 4047-4057.
Spradling, A. C. and Rubin, G. M. (1982). Transposition of cloned P-elements into Drosophila germ line chromosomes. *Science* **218**, 341-347.
Srinivasan, S., Rashka, K. E. and Bier, E. (2002). Creation of a sog morphogen gradient in the *Drosophila* embryo. *Dev. Cell* **2**, 91-101.
Stathopoulos, A. and Levine, M. (2002). Dorsal gradient networks in the *Drosophila* embryo. *Dev. Biol.* **246**, 57-67.
Tatei, K., Cai, H., Ip, Y. T. and Levine, M. (1995). *Race*: a *Drosophila* homologue of the angiotensin converting enzyme. *Mech. Dev.* **51**, 157-168.
Thor, S. and Thomas, J. B. (1997). The *Drosophila* islet gene governs axon pathfinding and neurotransmitter identity. *Neuron* **18**, 397-409.
Waltzer, L. and Bienz, M. (1999). A function of CBP as a transcriptional co-activator during Dpp signalling. *EMBO J.* **18**, 1630-1641.
Wharton, K. A., Ray, R. P. and Gelbart, W. M. (1993). An activity gradient of decapentaplegic is necessary for the specification of dorsal pattern elements in the *Drosophila* embryo. *Development* **117**, 807-822.
Wharton, S. S., Basu, S. P. and Ashe, H. L. (2004). Smad affinity can direct distinct readouts of the embryonic extracellular Dpp gradient in *Drosophila*. *Curr. Biol.* **14**, 1550-1558.
Winick, J., Abel, T., Leonard, M. W., Michelson, A. M., Chardon-Loriaux, I., Holmgren, R. A., Maniatis, T. and Engel, J. D. (1993). A GATA family transcription factor is expressed along the embryonic dorsoventral axis in *Drosophila melanogaster*. *Development* **119**, 1055-1065.
Xu, X., Yin, Z., Hudson, J. B., Ferguson, E. L. and Frasch, M. (1998). Smad proteins act in combination with synergistic and antagonistic regulators to target Dpp responses to the *Drosophila* mesoderm. *Genes Dev.* **12**, 2354-2370.
Yip, M. L., Lamka, M. L. and Lipshitz, H. D. (1997). Control of germ-band retraction in *Drosophila* by the zinc-finger protein Hindsight. *Development* **124**, 129-141.
Zhang, H., Levine, M. and Ashe, H. L. (2001). Brinker is a sequence-specific transcriptional repressor in the *Drosophila* embryo. *Genes Dev.* **15**, 261-266.
Zusman, S. B., Sweeton, D. and Wieschaus, E. (1988). short gastrulation, a mutation causing delays in stage-specific cell shape changes during gastrulation in *Drosophila melanogaster*. *Dev. Biol.* **129**, 417-427.