An Analysis Using the *hobo* Genetic System Reveals That Combinatorial Signaling by the Dpp and Wg Pathways Regulates *dpp* Expression in Leading Edge Cells of the Dorsal Ectoderm in *Drosophila melanogaster*

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ABSTRACT

Our laboratory has contributed to the development of a genetic system based upon the *hobo* transposable element in *Drosophila melanogaster*. We recently reported that *hobo*, like the better-known *P* element, is capable of local transposition. In that study, we mobilized a *hobo* enhancer trap vector and generated two unique alleles of *decapentaplegic* (*dpp*), a transforming growth factor-β family member with numerous roles during development. Here we report a detailed study of one of those alleles (dpp^{F11}). To our knowledge, this is the first application of the *hobo* genetic system to understanding developmental processes. First, we demonstrate that lacZ expression from the dpp^{F11} enhancer trap accurately reflects *dpp* mRNA accumulation in leading edge cells of the dorsal ectoderm. Then we show that combinatorial signaling by the Wingless (Wg) pathway, the Dpp pathway, and the transcriptional coactivator Nejire (CBP/p300) regulates dpp^{F11} expression in these cells. Our analysis of dpp^{F11} suggests a model for the integration of Wg and Dpp signals that may be applicable to other developmental systems. Our analysis also illustrates several new features of the *hobo* genetic system and highlights the value of *hobo*, as an alternative to *P*, in addressing developmental questions.

TRANSPOSABLE elements are invaluable tools for genetic analysis in many organisms. Experimental systems have been developed around *P* and *hobo* elements in *Drosophila melanogaster*. Structurally similar, the genetic systems of these elements share many characteristics. For example, both *P* and *hobo* systems are capable of efficient germline transformation (Blackman *et al.* 1987), enhancer trapping mutagenesis (Smith *et al.* 1993), and local transposition (Newfeld and Takaesu 1999). However, the *hobo* system is not as well developed as the *P* system. Here we report new features of the *hobo* system and describe the first use of this system as an analytical tool to address topical issues in developmental genetics.

Two techniques that we discuss have been reported once previously but not in the context of developmental genetic analyses: plasmid rescue of genomic sequences flanking *hobo* transgene insertions and the analysis of β-galactosidase expression from *hobo* enhancer traps in embryos (Smith *et al.* 1993). Two related techniques are described for the first time: *hobo*-specific primers for sequencing flanking genomic DNA and the analysis of β-galactosidase expression from *hobo* enhancer traps in imaginal discs. Two larger issues related to the overall versatility of the *hobo* system are discussed: the stability of *hobo* transgenes in lab stocks and during crossing schemes and the feasibility of identifying enough suitable laboratory strains to conduct a thorough developmental genetics study.

As a point of departure we employed a unique allele of *decapentaplegic* (dpp^{F11}) generated in our local jumping study (Newfeld and Takaesu 1999). *dpp* is a well-characterized signaling molecule in the transforming growth factor-β family (TGF-β; Newfeld *et al.* 1999). Dpp plays many roles in Drosophila development, including the specification of dorsal ectoderm during early stages of embryogenesis (Ray *et al.* 1991). The Dpp signal transduction pathway includes two cytoplasmic Smad proteins, Mothers against dpp (Mad) and Medea (Med). In response to a Dpp signal, a multimeric Mad/Med complex enters the nucleus and participates in the transcription of specific genes (Wrana 2000).

For some developmental decisions, Dpp signals are sufficient to specify the proper cell fate. However, Dpp alone can be insufficient to specify the appropriate cell type. In these cases, combinatorial signaling by several pathways appears to be required for correct cell fate specification. For example, the Dpp and Wingless (Wg) pathways are required to specify cell fates along the dorsal-ventral axis in the adult abdomen (Kopp *et al.* 1999) and along the proximal-distal axis in the leg (Lecuit and Cohen 1997).

wg is a well-characterized Wg/int-1 (Wnt) family member in Drosophila (Shulman *et al.* 1998). Wg plays many developmental roles, including the specification of segment polarity during early embryogenesis (Baker

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1987). The Wg signal transduction pathway includes a cytoplasmic protein complex made up of several proteins including Armadillo (Arm, homologous to vertebrate β-catenin; Peifer and Wieschaus 1990). In response to a Wg signal, Arm is released from this complex, enters the nucleus, and participates in the transcription of specific genes (Polakis 2000).

Two studies have examined the mechanism of combinatorial signaling by TGF-β and Wnt pathways. Both studies focus on Smad proteins and Arm/β-catenin in Xenopus. In one study, coinjection of Smad2 and β-catenin activated the transcription of *siamois*, a common target gene, significantly above the levels of Smad2 or β-catenin alone (Crease *et al.* 1998). In the second study, complexes containing Smad4 and β-catenin synergistically affect the transcription of *twin*, a Wnt target gene (Nishita *et al.* 2000), suggesting that Smad4 participates in Wnt signaling. However, the authors are careful to say that no evidence exists for Med (homologous to vertebrate Smad4; Wisotzkey *et al.* 1998) activity in Wg signaling in Drosophila. How Wg and Dpp signals are integrated in Drosophila is currently unknown.

Here we address this question through a developmental genetic analysis of dpp^{F11}. We report that lacZ expression from dpp^{F11} accurately reflects *dpp* mRNA expression in leading edge cells of the dorsal ectoderm. Our analysis of dpp^{F11} suggests that combinatorial signaling by the Wg and Dpp pathways occurs via transcription factor complexes. Further, this study illustrates the value of the *hobo* genetic system for analyzing developmental mechanisms.

MATERIALS AND METHODS

Molecular biology: Plasmid rescue of genomic DNA flanking the H[Lw2] transgene in the *hobo* enhancer trap strain H[Lw2] dpp^{F11} Dp (2;2) DTD48 $dpp^{d\text{-}ho}/CyO$ was conducted as follows: 5′ flanking DNA was recovered by digestion with *Bam*HI (*Bam*HI cuts at nucleotide 10514 in the *dpp* sequence; GenBank accession no. U63857) and sequenced with primer pH 5 (5′-AATTGTAGGGTGTGAGTCGAGTG-3′); 3′ flanking DNA was recovered with *Hin*dIII (*Hin*dIII cuts at nucleotide 17688 in GenBank accession no. U63857) and sequenced with primer pH 6 (5′-ATCGGGTGGACGTAGAGTGCGAG-3′). Genomic Southerns to detect endogenous *hobo* elements were conducted as described (Blackman *et al.* 1987). A list of 78 strains analyzed for the presence of endogenous *hobo* elements is presented in Waldrip *et al.* (2001).

Fly stocks: The dpp^{F11} *hobo* enhancer trap strain is as described by Newfeld and Takaesu (1999). Two *armadillo* (*arm*) alleles: arm^2 (arm^{XM19}, moderate hypomorph) and arm^4 (arm^{YD35}, genetic null) are as described by Peifer and Wieschaus (1990). Two *nejire* (*nej*) alleles: nej^1 (strong hypomorph) and nej^3 (protein null) are as described by Akimaru *et al.* (1997). The arm^2 nej^3 and arm^2 FRT 101 strains are as described by Waltzer and Bienz (1998). The nej^1 FRT 101 strain is as described by Waltzer and Bienz (1998). The Med^1 (genetic null) strain is as described by Das *et al.* (1998). The *kayak* (*kay*) allele kay^1 (genetic null) is as described by Riesgo-Escovar and Hafen (1997). The blue balancer strains are as described: FM7c P[*eve*-lacZ] (Waltzer and Bienz 1998), *CyO* P[*wg*-lacZ] (Kassis *et al.* 1992), and TM3 P[*Scr*-lacZ] (Gindhart *et al.* 1995).

Genetics: All experimental chromosomes were maintained over blue balancers. In matings with dpp^{F11}, the *arm* and *nej* mutant strains (both genes are on the X chromosome) did not need to be *hobo*-free since mobilization of the transgene in the germline of experimental embryos was inconsequential. For tests of dpp^{F11} expression in *arm* and *nej* zygotic mutants, males carrying dpp^{F11} were crossed to females heterozygous for an *arm* allele (arm^2 or arm^4) or a *nej* allele (nej^1 or nej^3). For tests of dpp^{F11} expression in *Med* zygotic mutants, a double balanced stock was generated that carries dpp^{F11} and Med^1. A *hobo*-free Med^1 strain was used to construct this stock. No *hobo*-free *Mad* strains have been identified to date. For tests of dpp^{F11} expression in *kay* zygotic mutants, a double balanced stock was generated that carries dpp^{F11} and kay^1. A *hobo*-free kay^1 strain was used to construct this stock. For tests of dpp^{F11} expression in *arm nej* zygotic double mutants, males carrying dpp^{F11} were crossed to females heterozygous for an arm^2 nej^3 chromosome. For tests of dpp^{F11} expression in germline clone (GLC) mutant embryos (embryos lacking maternal and zygotic gene activity), females bearing GLC of arm^2 or nej^1 were mated to males carrying an X chromosome blue balancer and dpp^{F11}. The hypomorphic alleles arm^2 and nej^1 were used to make GLC because the null alleles arm^4 and nej^3 do not come through the germline (Peifer and Wieschaus 1990; Waltzer and Bienz 1998). Females bearing GLC were generated using the FLP-DFS system (Chou and Perrimon 1992). To determine whether Med^1 mutations dominantly enhance the effect of *arm* and *nej* mutations on dpp^{F11} expression, we generated arm^4 and nej^3 zygotic mutant embryos that were also heterozygous for Med^1. These embryos were derived from crosses between males that carried dpp^{F11} and Med^1 and females heterozygous for arm^4 or nej^3.

Gene expression: Histochemical staining for β-galactosidase (lacZ) activity in embryos was conducted as described by Newfeld *et al.* (1996). We utilize histochemical staining for the following reasons: (1) The strong catalytic ability of lacZ significantly amplifies weak signals (such as those seen in the germline clone embryos) well above that obtainable with antibodies to lacZ, and (2) histochemical staining is the only method capable of detecting lacZ activity after cuticle deposition during stage 16 (Ashburner 1989). For consistency, histochemical staining is reported for all embryos. Processing of all embryos shown in the same figure was conducted in parallel to minimize variation between staining reactions. Histochemical staining for β-galactosidase (lacZ) activity in imaginal discs was conducted as described by Marquez *et al.* (2001). RNA *in situ* hybridization with the *dpp* cDNA H1 was conducted as described by Ray *et al.* (1991).

RESULTS

dpp^{F11} lacZ expression accurately depicts *dpp mRNA* expression in leading edge cells of the dorsal ectoderm: dpp^{F11} is a unique haplolethal allele (maintained in stock with a duplication of *dpp*) that carries a *hobo* enhancer trap construct inserted in the *dpp* transcription unit. Restriction fragment length polymorphism data initially suggested that the dpp^{F11} transgene is inserted into intron 2 (Newfeld and Takaesu 1999). Subsequently, plasmid rescue of genomic sequences flanking the dpp^{F11} transgene showed a precise insertion between nucleotides 13434 and 13435 of the *dpp* genomic sequence (GenBank accession no. U63857). This places the inser-

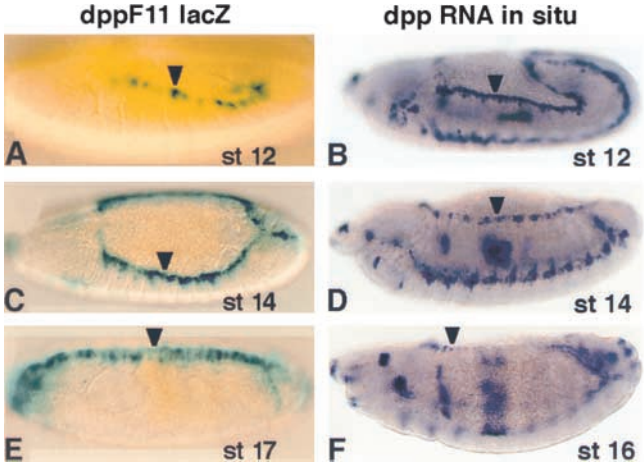

FIGURE 1.—Comparison of wild-type *dpp* mRNA and dpp^{F11} lacZ expression. Staged embryos are shown and arrowheads indicate expression in leading edge cells of the dorsal ectoderm. (A and B) dpp^{F11} lacZ and *dpp* mRNA are strongly expressed in leading edge cells. (C and D) dpp^{F11} lacZ and *dpp* mRNA expression in these cells is maintained at high levels through dorsal closure. (E and F) dpp^{F11} lacZ and *dpp* mRNA expression are visible in cells along the dorsal midline. dpp^{F11} lacZ expression accurately reflects *dpp* mRNA expression in leading edge cells of the dorsal ectoderm.

tion 40 nucleotides upstream of the exon 2 splice acceptor and between the codons for leucine 276 and threonine 277 in the *dpp* open reading frame. Given the proximity of the dpp^{F11} enhancer trap insertion to intron 2, we wondered if dpp^{F11} lacZ expression was under the influence of an intronic enhancer. Previous studies have shown that there are tissue-specific enhancers and repressors in intron 2 (HUANG *et al.* 1993) as well as numerous conserved sequences of unknown function (NEWFELD *et al.* 1997).

Histochemical examination of embryos revealed that the dpp^{F11} transgene expresses lacZ exclusively in leading edge cells of the dorsal ectoderm. lacZ expression begins during germband retraction (stage 12, Figure 1A) and continues strongly during the leading edge cell movements known as dorsal closure (stage 14, Figure 1C). After dorsal closure, leading edge cells from both sides of the embryo form the dorsal midline and dpp^{F11} expression is still strong (stage 17, Figure 1E). A side-by-side comparison shows that lacZ expression from the dpp^{F11} transgene accurately reflects *dpp* mRNA expression in leading edge cells (Figure 1, B, D, and F). This is true up to the limit of detection for RNA *in situ* hybridization experiments (stage 16, due to cuticle deposition).

We also examined dpp^{F11} lacZ expression in wing and leg imaginal discs. We did not detect any expression in leg discs. In wing discs, dpp^{F11} expression was visible just anterior to the anterior-posterior compartment boundary (data not shown) in a pattern that accurately reflects *dpp* expression (BLACKMAN *et al.* 1991).

The correspondence of *dpp* mRNA expression and lacZ expression from dpp^{F11} in leading edge cells suggested that an analysis of dpp^{F11} regulation would reveal factors regulating *dpp* mRNA expression in this tissue. Given *dpp*'s highly dynamic expression pattern, the ability to focus on the regulation of just one aspect of *dpp* expression using the dpp^{F11} enhancer trap simplifies the analysis tremendously. dpp^{F11} is the only transgene that mimics just this aspect of *dpp* expression. The region where dpp^{F11} is inserted is refractory to *P*-element enhancer trap insertion (NEWFELD and TAKAESU 1999) and *dpp* leading edge expression is not recapitulated by any existing reporter gene (FLYBASE 2002). Finally, the regulatory sequences that drive *dpp* mRNA expression in leading edge cells have not yet been identified. Thus, the dpp^{F11} *hobo* transgene insertion appears to provide a unique opportunity to further illuminate mechanisms of *dpp* regulation in leading edge cells.

dpp^{F11} expression is not fully maintained in *kay*, *arm*, *Med*, or *nej* zygotic mutants: If studies of dpp^{F11} regulation are to provide new insight into the regulation of *dpp* mRNA expression in leading edge cells, then dpp^{F11} must mimic *dpp* mRNA expression in wild-type and mutant embryos. To test this premise, we analyzed dpp^{F11} expression in Jun amino-terminal kinase (JNK), Wg and Dpp signaling pathway mutants with demonstrated effects on *dpp* mRNA expression. We examined embryos with zygotic mutations in the following genes: *kay* (dFos), a transcription activator in the JNK pathway; *arm*, a transcription activator in the Wg pathway; and *Med*, a transcription activator in the Dpp pathway. *dpp* mRNA expression in leading edge cells is not maintained in *kay* mutants (RIESGO-ESCOVAR and HAFEN 1997), *arm* mutants (MCEWEN *et al.* 2000), or Dpp pathway mutant embryos (TORRES-VAZQUEZ *et al.* 2001). If dpp^{F11} expression is an accurate reflection of *dpp* mRNA expression, then dpp^{F11} expression should not be maintained in leading edge cells in these mutants.

In embryos younger than stage 15, we observed relatively normal expression of dpp^{F11} in each mutant background. This is likely due to the fact that *kay*, *arm*, and *Med* have a maternal component that sustains dpp^{F11} expression in these embryos (FLYBASE 2002). In late-stage embryos, dpp^{F11} expression in leading edge cells was well below wild-type levels in kay^1, arm^4, and Med^1 null mutant backgrounds (Figure 2, A, B, and D). In stage 17 embryos, each mutant's effect on dpp^{F11} expression matches the severity of its mutant phenotype. kay^1 and arm^4 zygotic mutants have "dorsal open" phenotypes with extensive defects in tissues derived from the dorsal ectoderm (PEIFER and WIESCHAUS 1990; RIESGO-ESCOVAR and HAFEN 1997). Dorsal defects are seen only occasionally in Med^1 zygotic mutants (S. NEWFELD, unpublished observations). The data for dpp^{F11} agree with previous studies that showed that the JNK pathway, the Wg pathway, and the Dpp pathway are required to maintain *dpp* mRNA expression in leading edge cells. This

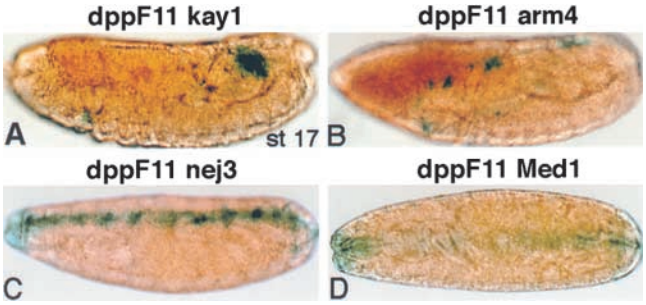

FIGURE 2.—dpp^{F11} expression is not fully maintained in *kay*, *arm*, *nej*, or *Med* zygotic mutants. Stage 17 embryos are shown. lacZ expression from dpp^{F11} is shown in kay^{1} (A), arm^{4} (B), nej^{3} (C), and Med^{1} zygotic mutant embryos (D). dpp^{F11} expression is below wild-type levels (see Figure 1E) in all embryos.

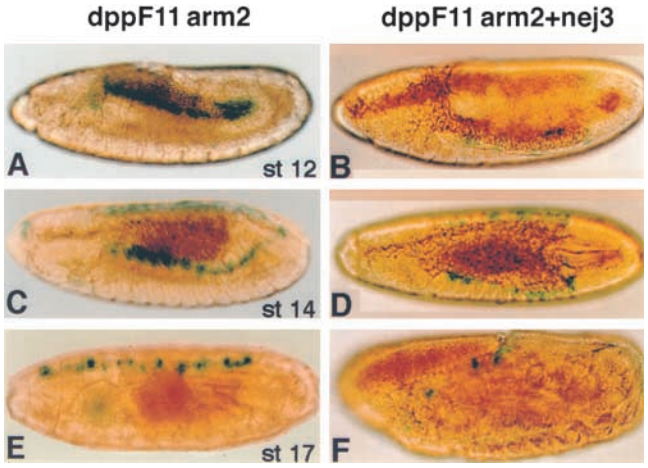

FIGURE 3.—An *arm nej* zygotic double mutant shows synergystic effects on dpp^{F11} expression. Staged embryos are shown. lacZ expression from dpp^{F11} is shown in arm^{2} zygotic mutants (A, C, and E) and arm^{2} nej^{3} zygotic double-mutants embryos (B, D, and F). The effect of arm^{2} nej^{3} zygotic double mutants on dpp^{F11} initiation and maintenance is much more severe than that seen in arm^{2} or nej^{3} (see Figure 2C) zygotic single mutants.

correspondence supports the use of dpp^{F11} in further studies of Wg and Dpp pathway regulation of *dpp* expression in leading edge cells.

We then examined lacZ expression from dpp^{F11} in *nej* zygotic mutant embryos. *dpp* mRNA expression in leading edge cells has not been studied in *nej* mutants. *nej* is the Drosophila homolog of the mammalian transcription coactivator CBP/p300 (AKIMARU *et al.* 1997). We utilized *nej* mutants for two reasons. First, two studies have shown that *nej* can participate in the Dpp signaling pathway. Expression from a Dpp-responsive midgut enhancer is reduced in nej^{3} zygotic mutant embryos (WALTZER and BIENZ 1999) and dorsal-ventral patterning genes requiring maximal levels of Dpp signaling (*e.g.*, *hindsight*) are not expressed in nej^{1} germline clone mutants (ASHE *et al.* 2000). Second, *nej* was shown to antagonize Wg signaling in the midgut mesoderm (WALTZER and BIENZ 1998). If the Dpp pathway and the Wg pathway are both required for dpp^{F11} expression in leading edge cells, then we wondered if Nej (to our knowledge, the only gene shown to influence both pathways) was somehow involved.

In nej^{3} null mutants, we observed relatively normal expression of dpp^{F11} in embryos younger than stage 15 because *nej* also has a maternal component (AKIMARU *et al.* 1997). In stage 17 embryos, dpp^{F11} expression in the leading edge was below wild-type levels in nej^{3} null mutants (Figure 2C). In these embryos, *nej*'s effect on dpp^{F11} expression matches the severity of its mutant phenotype. Dorsal ectoderm defects are seen only rarely in nej^{3} zygotic mutants (M. BIENZ, personal communication). Overall, the zygotic mutant data suggest that the JNK pathway via *kay*, the Wg pathway via *arm*, and the Dpp pathway via *Med* and *nej* are all required to maintain dpp^{F11} expression in leading edge cells.

An *arm nej* zygotic double mutant shows synergystic effects on dpp^{F11} expression: Interestingly, *arm* and *nej* zygotic mutants both reduce the level of dpp^{F11} expression. In leading edge cells, *nej* does not appear to antagonize Wg signaling as it does in the midgut mesoderm (WALTZER and BIENZ 1998). A positive role for *nej* in Wg signaling has not been shown previously in Drosophila. This possibility does have a precedent in vertebrates. In Xenopus, CBP (*nej* homolog) synergized with β-catenin (*arm* homolog) to stimulate the transcription of Wnt target genes (TAKEMARU and MOON 2000). Alternatively, the reduction in dpp^{F11} expression in *nej* mutants may be due to *nej* playing a positive role in Dpp signaling. To date, *nej* has not been reported to participate in the JNK pathway and we have preliminary data, discussed below, suggesting that JNK regulation of *dpp* expression in leading edge cells is independent of the Wg and Dpp pathways.

We tested the hypothesis that Nej plays a positive role in the Wg signaling pathway in the regulation of *dpp* expression in leading edge cells. We examined dpp^{F11} expression in arm^{2} nej^{3} zygotic double-mutant embryos and looked for additive effects. arm^{2} is a moderate hypomorphic allele and arm^{2} zygotic mutant embryos do not have dorsal defects (PEIFER and WIESCHAUS 1990). We reasoned that if *arm* and *nej* were acting synergistically in the Wg pathway, then the effect of the zygotic double mutant would be more severe than that of either zygotic single mutant alone. Alternatively, if *nej* were acting in the Dpp pathway, then the effect of the double mutant should be similar to the effect of each single mutant.

dpp^{F11} expression is affected much more severely in an arm^{2} nej^{3} zygotic double mutant than in either single mutant. In the double mutant, dpp^{F11} expression is virtually absent in late-stage embryos (Figure 3F) whereas dpp^{F11} expression is clearly visible in arm^{2} (Figure 3E) and nej^{3} (Figure 2C) single mutants. The presence of nej^{3} clearly enhances (not antagonizes) the effect of arm^{2} on dpp^{F11} expression in double-mutant embryos.

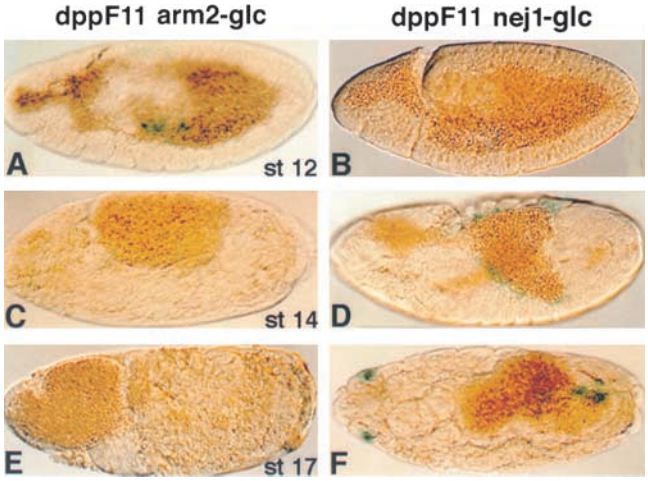

FIGURE 4.—*dpp*^{F11} expression does not initiate properly in *arm* or *nej* GLC mutants. Staged, hemizygous GLC mutant embryos (those without maternal or zygotic gene function) are shown. lacZ expression from *dpp*^{F11} is shown in *arm*^{2} GLC (A, C, and E) and *nej*^{1} GLC mutant embryos (B, D, and F). The effect of *arm*^{2} and *nej*^{1} GLC mutants on *dpp*^{F11} expression is more severe than that of *arm*^{2} *nej*^{3} zygotic double-mutant embryos (see Figure 3, B, D, and F).

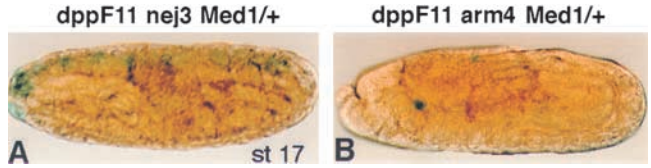

FIGURE 5.—Dominant enhancement of *arm* and *nej* zygotic mutants by *Med*^{1}. Stage 17 embryos are shown. lacZ expression from *dpp*^{F11} is shown in *nej*^{3} (A) and *arm*^{4} zygotic mutant embryos (B) that are also heterozygous for *Med*^{1}. Heterozygosity for *Med*^{1} significantly enhances the effect of *nej*^{3} (see Figure 2C) and *arm*^{4} (see Figure 2B) zygotic mutants on *dpp*^{F11} expression.

This synergistic effect, the significant reduction of *dpp*^{F11} expression in *arm*^{2} *nej*^{3} zygotic double mutants, supports the hypothesis that *nej* acts positively in the Wg pathway to maintain *dpp*^{F11} expression.

We also noted that *dpp*^{F11} expression does not initiate at wild-type levels in *arm*^{2} *nej*^{3} zygotic double mutants and expression remains below wild-type levels even in mid-stage embryos (Figure 3, B and D). In *arm*^{2} embryos younger than stage 15, we observed relatively normal expression of *dpp*^{F11} (Figure 3, A and C). The initiation of *dpp*^{F11} expression may be affected in double-mutant embryos because the female parent is heterozygous for the double-mutant chromosome. Heterozygosity of the female parent for *arm*^{2} or *nej*^{3} single-mutant chromosomes had no effect on *dpp*^{F11} initiation in these mutant embryos. Again, the presence of *nej*^{3} enhances (not antagonizes) the effect of *arm*^{2} on *dpp*^{F11} expression in double-mutant embryos. This second synergistic effect, the inability to fully initiate *dpp*^{F11} expression, suggests that *arm* and *nej* as part of the Wg pathway are required for the initiation of *dpp* expression in leading edge cells.

***dpp*^{F11} expression does not properly initiate in *arm* or *nej* GLC mutants:** We tested the hypothesis that *arm* and *nej* are required for the initiation of *dpp*^{F11} expression. We examined embryos lacking maternal and zygotic gene function derived from females bearing *arm*^{2} or *nej*^{1} GLC. The hypomorphic alleles *arm*^{2} and *nej*^{1} were used to make GLC because the null alleles *arm*^{4} and *nej*^{3} do not come through the germline (PEIFER and WIESCHAUS 1990; WALTZER and BIENZ 1998).

Weak *dpp*^{F11} expression is seen at stage 12 in *arm*^{2} GLC embryos (Figure 4A). No lacZ expression is seen at later stages in *arm*^{2} GLC mutant embryos (Figure 4, C and E). *dpp*^{F11} expression does not initiate during stage 12 in *nej*^{1} GLC mutant embryos (Figure 4B). Faint lacZ expression is seen at later stages in *nej*^{1} GLC mutant embryos (Figure 4, C and F). *dpp*^{F11} expression in these embryos is likely due to the fact that *arm*^{2} and *nej*^{1} are not null alleles. In stage 17 embryos, each mutant's effect on *dpp*^{F11} expression matches the severity of its mutant phenotype. *nej*^{1} GLC and *arm*^{2} GLC mutant embryos have extensive dorsal defects (PEIFER and WIESCHAUS 1990; ASHE *et al.* 2000). Taken together, our analyses of three classes of *arm* and *nej* mutants (zygotic single, zygotic double, and GLC) suggest that the Wg pathway is required for the initiation and maintenance of *dpp* expression in leading edge cells.

***Med*^{1} is a dominant enhancer of *arm*^{4} and *nej*^{3} effects on *dpp*^{F11} expression:** We formally tested the hypothesis that the Wg pathway and the Dpp pathway act synergistically in the maintenance of *dpp* expression in leading edge cells. We assayed for dominant interactions between components of these pathways. Specifically, we examined lacZ expression from *dpp*^{F11} in *arm*^{4} or *nej*^{3} zygotic mutant embryos that were also heterozygous for *Med*^{1}. We reasoned that if the two pathways were acting independently, then heterozygosity for *Med*^{1} (a recessive null allele) would have no effect on *arm*^{4} or *nej*^{3} regulation of *dpp*^{F11} expression. However, if there were a synergistic interaction between the pathways, then the dosage of *Med* could influence the affect of *arm*^{4} or *nej*^{3} on the maintenance of *dpp*^{F11} expression.

The initiation of lacZ expression from *dpp*^{F11} in leading edge cells is largely unaffected in all embryos due to maternal contributions from each gene. However, *dpp*^{F11} expression is well below wild-type levels in both *Med*-enhanced zygotic mutant backgrounds at stage 17 (compare Figure 5, A and B, with Figure 1E). Of greater importance, the effect of *arm*^{4} or *nej*^{3} on *dpp*^{F11} expression is more severe in the absence of one functional copy of *Med* than in their respective zygotic single mutants. To see the effect of heterozygosity for *Med*^{1}, compare Figure 5A to Figure 3C for *nej*^{3} and compare Figure 5B to Figure 2B for *arm*^{4}. Dominant enhancement of *arm*^{4} and *nej*^{3} zygotic mutant phenotypes by *Med*^{1} strongly suggests that the Dpp pathway synergizes with the Wg pathway

to maintain *dpp* expression in leading edge cells. Further, the data indicate that the transcriptional coactivator Nej, with its positive roles in both Wg signaling (Figure 3) and Dpp signaling (Waltzer and Bienz 1999), may act to bridge the pathways.

DISCUSSION

During early stages of embryogenesis, *wg* and *dpp* are expressed in undifferentiated dorsal ectoderm. *wg* mRNA expression, in 15 stripes along the entire dorsal-ventral axis of the embryo (including the dorsal ectoderm), begins at stage 8. *wg* expression persists in this striped pattern through stage 17 (Baker 1987). *dpp* mRNA is expressed on the dorsal side of the embryo along the entire anterior-posterior axis, beginning at stage 4. *dpp* mRNA expression persists in a large portion of the dorsal ectoderm through stage 8 and resolves into leading edge cell-specific expression in stage 12 embryos (Ray *et al.* 1991). At this time the embryonic expression pattern of *nej* has not been reported. However, some information can be inferred from *nej* mutant phenotypes. *nej* zygotic mutant embryos show visible defects in the tracheal system at stage 12 (Waltzer and Bienz 1999). The tracheal system is derived from the dorsal ectoderm, suggesting that *nej* is expressed in this tissue prior to stage 12.

Our analysis of dpp^{F11} suggests that *dpp* expression in leading edge cells is initiated by prior episodes of *wg* and *dpp* expression in the undifferentiated dorsal ectoderm. The maintenance of *dpp* expression in leading edge cells appears to require continuous input from *wg* and from a *dpp* feedback loop. The initiation and maintenance of *dpp* expression in leading edge cells also require continuous *nej* activity. Overall, our data are consistent with the following combinatorial signaling model (Figure 6): Med (signaling for the Dpp pathway) interacts with Arm (signaling for the Wg pathway) via the transcriptional coactivator Nej. This multimeric complex initiates and, with continuous signaling, maintains *dpp* expression in leading edge cells.

Our data extend previous studies of *dpp* expression in leading edge cells and Dpp signaling in several ways. McEwen *et al.* (2000) suggest a role for Wg signaling in the regulation of *dpp* expression in the leading edge. Their data are consistent with ours. We both show that *dpp* leading edge expression is not maintained in arm^2 zygotic mutants and does not initiate in arm^2 germline clones. We extend their study by demonstrating the involvement of *nej* and *Med* in the regulation of *dpp* expression in leading edge cells. Waltzer and Bienz (1999) report that *nej* participates in Dpp signaling. Their data are consistent with ours. While they show that nej^3 enhances *dpp* wing phenotypes, we show that Med^1 enhances nej^3 embryonic phenotypes. We extend their study by suggesting a role for *nej* in mediating combinatorial signaling by the Wg and Dpp pathways.

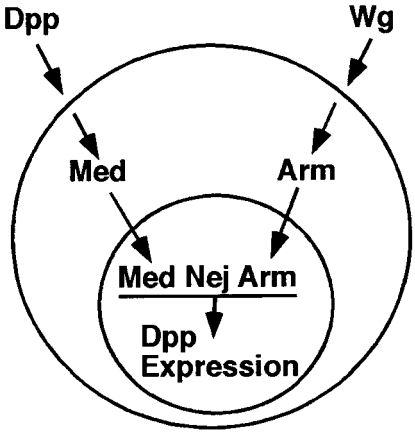

Figure 6.—Combinatorial model for the regulation of *dpp* expression in leading edge cells. In leading edge cells, Dpp signals are carried from the cytoplasm to the nucleus by a complex that includes Med. Wg signals are carried by a complex that includes Arm. In the nucleus, Dpp and Wg signals are integrated via a multimeric transcription factor complex that includes Arm, Med, and the coactivator Nej. This complex initiates *dpp* expression. With continuous Wg and Dpp signaling, complexes of this type are constantly formed and they act to maintain *dpp* expression in these cells.

Several questions remain about the combinatorial regulation of *dpp* expression by Wg, Dpp, and Nej. One question is, how is *Nej* recruited to bridge the two pathways? Numerous studies have shown that p300/CBP transcriptional coactivation functions are stimulated by its phosphorylation but the site of phosphorylation has never been mapped (Goodman and Smolik 2000). Perhaps Zeste white3 (a serine-threonine kinase in the Wg pathway) or Thickveins (a serine-threonine kinase in the Dpp pathway) are involved in recruiting Nej to participate in combinatorial signaling.

A second question concerns the nature of the enhancer element that directs *dpp* expression in leading edge cells. Using reporter genes, we have identified a 54-nucleotide candidate enhancer near the dpp^{F11} transgene insertion that drives lacZ expression in a subset of leading edge cells (Takaesu *et al.* 2002). The region contains two sets of conserved, overlapping consensus-binding sites for dTCF (a transcriptional partner for Arm in the Wg pathway) and Mad (a transcriptional partner for Med in the Dpp pathway). No JNK-pathway-binding sites are in the region, suggesting that JNK regulation of *dpp* expression in leading edge cells is independent of Wg and Dpp signaling.

Interestingly, there is also a consensus Brinker (Brk) binding site in the candidate enhancer (Rushlow *et al.* 2001). Brk is a transcriptional repressor of Dpp target genes and one mechanism by which Dpp signaling activates its target genes is to relieve Brk repression (Torres-Vazquez *et al.* 2001). Our genetic data cannot discriminate between the possibility that combinatorial signaling by the Wg and Dpp pathways regulates *dpp*

expression in leading edge cells by direct activation or by relief of Brk repression. Using this candidate enhancer, we are preparing to conduct biochemical analyses of DNA-protein interactions that will determine if one or both of these mechanisms are involved.

In addition to advancing our understanding of *dpp* regulation in leading edge cells, our analysis of dpp^{F11} further establishes the value of the *hobo* genetic system as an analytical tool in Drosophila. Our study shows that (with the caveat that suitable strains must first be identified) the *hobo* system is capable of a wide range of sophisticated genetic techniques first developed for the *P*-element system. We demonstrate several technical advances for the *hobo* genetic system that reflect its versatility. This study is the first to utilize plasmid rescue of sequences flanking *hobo* transgenes and the histochemical analysis of β-galactosidase expression from *hobo* enhancer trap vectors in embryos as analytical tools to address developmental questions. In addition, we describe a set of *hobo* sequencing primers for the analysis of rescued, flanking genomic DNA and the analysis of β-galactosidase expression from *hobo* enhancer traps in imaginal discs.

Like many genetic analyses, our study of dpp^{F11} was conducted over several years. This allows us to address important issues about the long-term stability of *hobo* transgenes in permanent laboratory stocks and during complex crossing schemes as well as the practicality of finding suitable strains for the analysis of one's favorite *hobo*-associated mutant. Regarding the stability of *hobo* transgenes in stocks and in crosses, we found absolutely no evidence of instability. In our hands, this issue is no more relevant for *hobo* than it is for *P*. The dpp^{F11} strain has been successfully maintained in stock for nearly a decade side by side with *P* transgene strains. During this time there were no alterations to the genetic or molecular characteristics of the dpp^{F11} strain. For example, the strain always demonstrates haploinsufficiency when recombinant progeny with the *hobo* insertion but without the *dpp* duplication are generated and there have never been any alterations in eye color or lacZ expression pattern.

Regarding the practicality of finding suitable strains for the analysis of one's favorite *hobo*-associated mutant, we admit that this is more tedious than using the *P* system. The trade-off is that *P* and *hobo* elements have distinct insertion preferences. This was shown in a genome-wide survey (Smith *et al.* 1993) and in an analysis at the *dpp* locus (Newfeld and Takaesu 1999). In addition, there is no *a priori* reason to believe that strains associated with Dpp signaling, such as those used in this study, are more or less prone to possess endogenous *hobo* elements than those necessary for the analysis of other genes. Thus, it seems likely that suitable strains can be found for just about any study. We are willing to share strains that are widely applicable for *hobo* genetics, such as those useful for germline transformation, mutagenesis screens, and blue balancers. See Waldrip *et al.* (2001) for a complete list of available strains and a discussion of two large collections of developmental mutants compatible with the *hobo* genetic system.

From a genome-wide perspective, the majority of predicted genes are not yet mutagenized by *P*-element insertions (Spradling *et al.* 1999). Some well-studied genes appear immune to such insertions. For example, no *P*-element mutations were found in alcohol dehydrogenase in a database search that identified 106 mutant alleles (Ashburner *et al.* 1999). Thus, it seems logical to utilize another element with a well-developed genetic system such as *hobo* to extend the reach of current mutagenesis methods. It seems likely that the *hobo* enhancer trap collection of Smith *et al.* (1993), which has not been widely exploited for genetic analyses, contains hits in genes not susceptible to *P* insertion.

In summary, our study suggests that an expanded use of *hobo* transgenes will facilitate our understanding of the developmental biology of *D. melanogaster*. Given their membership in large multigene families, our analysis of the combinatorial regulation of dpp^{F11} expression in leading edge cells by Dpp and Wg will likely have wide relevance to TGF-β and Wnt signaling in many species.

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