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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Manuscript received November 27, 2001
Accepted for publication March 7, 2002

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 (dppF11). 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 dppF11 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 dppF11 expression in these cells. Our analysis of dppF11 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.

TRANPOSABLE 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 (dppF11) 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).

wag 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 et al. 1999).
1987). The Wg signal transduction pathway includes a cytoplasmic protein complex made up of several proteins including Armadillo (Arm, homologous to vertebrate β-catenin; PEIFFER and WIECHSCHAU 1990). In response to a Wg signal, Arm is released from this complex, enters the nucleus, and participates in the transcription of specific genes (POLARIS 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 dppβ11. We report that lacZ expression from dppβ11 accurately reflects dpp mRNA expression in leading edge cells of the dorsal ectoderm.

Our analysis of dppβ11 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/H2 transgene in the hobo enhancer trap strain H/H2 dppβ11 Dp (2;2) DTD48 dpp+/CyO was conducted as follows: 5′ flanking DNA was recovered by digestion with BamHI (BamHI cuts at nucleotide 10514 in the dpp sequence; GenBank accession no. U63857) and sequenced with primer pH5 (5′-AATTGTAGGGTGTGAGTCGAGTG-3′); 3′ flanking DNA was recovered with HindIII (HindIII cuts at nucleotide 17688 in GenBank accession no. U63857) and sequenced with primer pH6 (5′-ATCGGTTGGACTAGTGGCAG-3′). Genetic Southern 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 dppβ11 hobo enhancer trap strain is as described by NEWFELD and TAKAE SU (1999). Two armadillo (arm) alleles: armβ (armMOX, moderate hypomorph) and armβ (armMOX, genetic null) are as described by PEIFFER and WIECHSCHAU (1990). Two nej1 (nej1, strong hypomorph) and nej2 (protein null) are as described by ARIMARU et al. (1997). The armβ nej1 and armβ FRT 101 strains are as described by WALTZER and BIEZNS (1998). The nej1 FRT 101 strain is as described by WALTZER and BIEZNS (1998). The Med (genetic null) strain is as described by DAS et al. (1998). The kayak (kay) allele kayβ (genetic null) is as described by RIESGO-ESCOVAR and HAFEN (1997). The blue balanced strains are as described; FM7c P[neo-lacZ] (WALTZER and BIEZNS 1998), CyO P[ug-lacZ] (KASSIS et al. 1992), and TM3 P[So-lacZ] (GINDHART et al. 1995).

Genetics: All experimental chromosomes were maintained over blue balancers. In matings with dppβ11, 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 dppβ11 expression in arm and nej zygotic mutants, males carrying dppβ11 were crossed to females heterozygous for an arm allele (armβ or armβ) or a nej allele (nej1 or nej2). For tests of dppβ11 expression in Med zygotic mutants, a double balanced stock was generated that carries dppβ11 and Med. A hobo-free Med strain was used to construct this stock. No hobo-free Med strains have been identified to date. For tests of dppβ11 expression in any zygotic mutants, a double balanced stock was generated that carries dppβ11 and kayβ. A hobo-free kayβ strain was used to construct this stock. For tests of dppβ11 expression in arm nej zygotic double mutants, males carrying dppβ11 were crossed to females heterozygous for an armβ nej3 chromosome. For tests of dppβ11 expression in germline clone (GLC) mutant embryos (embryos lacking maternal and zygotic gene activity), females bearing GLC of armβ or nej null were mated to males carrying an X chromosome blue balancer and dppβ11. The hypomorphic alleles armβ and nej were used to make GLC because the null alleles armβ and nej do not come through the germline (PEIFFER and WIECHSCHAU 1990; WALTZER and BIEZNS 1998). Females bearing GLC were generated using the FLP-DHS system (CHOU and PERRIMON 1992). To determine whether Med1 mutations dominantly enhance the effect of arm and nej mutations on dppβ11 expression, we generated armβ and nej1 zygotic mutant embryos that were also heterozygous for Med1. These embryos were derived from crosses between males that carried dppβ11 and Med1 and females heterozygous for armβ or nej1.

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
dppβ11 lacZ expression accurately depicts dpp mRNA expression in leading edge cells of the dorsal ectoderm: dppβ11 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 dppβ11 transgene is inserted into intron 2 (NEWFELD and TAKAE SU 1999). Subsequently, plasmid rescue of genomic sequences flanking the dppβ11 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 dppF11 lacZ expression. Staged embryos are shown and arrowheads indicate expression in leading edge cells of the dorsal ectoderm. (A and B) dppF11 lacZ and dpp mRNA are strongly expressed in leading edge cells. (C and D) dppF11 lacZ, and dpp mRNA expression in these cells is maintained at high levels through dorsal closure. (E and F) dppF11 lacZ, and dpp mRNA expression are visible in cells along the dorsal midline. dppF11 lacZ expression accurately reflects dpp mRNA expression in leading edge cells of the dorsal ectoderm.

The correspondence of dpp mRNA expression and lacZ expression from dppF11 in leading edge cells suggested that an analysis of dppF11 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 dppF11 enhancer trap simplifies the analysis tremendously. dppF11 is the only transgene that mimics just this aspect of dpp expression. The region where dppF11 is inserted is refractory to Pelement 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 dppF11 hobo transgene insertion appears to provide a unique opportunity to further illuminate mechanisms of dpp regulation in leading edge cells.

dppF11 expression is not fully maintained in kay, arm, Med, or ney zygotic mutants: If studies of dppF11 regulation are to provide new insight into the regulation of dpp mRNA expression in leading edge cells, then dppF11 must mimic dpp mRNA expression in wild-type and mutant embryos. To test this premise, we analyzed dppF11 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 dppF11 expression is an accurate reflection of dpp mRNA expression, then dppF11 expression should not be maintained in leading edge cells in these mutants.

In embryos younger than stage 15, we observed relatively normal expression of dppF11 in each mutant background. This is likely due to the fact that kay, arm, and Med have a maternal component that sustains dppF11 expression in these embryos (FlyBase 2002). In late-stage embryos, dppF11 expression in leading edge cells was well below wild-type levels in kay', arm', and Med' null mutant backgrounds (Figure 2, A, B, and D). In stage 17 embryos, each mutant’s effect on dppF11 expression matches the severity of its mutant phenotype. kay', arm', and Med' 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' zygotic mutants (S. Newfeld, unpublished observations). The data for dppF11 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
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 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 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 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 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 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 synergistic 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 nej zygotic double-mutant embryos and looked for additive effects. arm is a moderate hypomorphic allele and arm 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 nej 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 mutant (Figure 3E) and nej5 (Figure 2C) single mutants. The presence of nej5 clearly enhances (not antagonizes) the effect of arm on $dpp^{F11}$ expression in double-mutant embryos.

**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 (A), arm (B), nej (C), and Med 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 synergistic effects on $dpp^{F11}$ expression. Staged embryos are shown. lacZ expression from $dpp^{F11}$ is shown in arm nej zygotic mutants (A, C, and E) and arm nej zygotic double-mutant embryos (B, D, and F). The effect of arm nej zygotic double mutants on $dpp^{F11}$ initiation and maintenance is much more severe than that seen in arm or nej (see Figure 2C) zygotic single mutants.
This synergistic effect, the significant reduction of dppF11 expression in arm2 nej3 zygotic double mutants, supports the hypothesis that nej acts positively in the Wg pathway to maintain dppF11 expression.

We also noted that dppF11 expression does not initiate at wild-type levels in arm2 nej3 zygotic double mutants and expression remains below wild-type levels even in mid-stage embryos (Figure 3, B and D). In arm2 embryos younger than stage 15, we observed relatively normal expression of dppF11 (Figure 3, A and C). The initiation of dppF11 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 arm2 or nej1 single-mutant chromosomes had no effect on dppF11 initiation in these mutant embryos. Again, the presence of nej3 enhances (not antagonizes) the effect of arm2 on dppF11 expression in double-mutant embryos. This second synergistic effect, the inability to fully initiate dppF11 expression, suggests that arm and nej as part of the Wg pathway are required for the initiation of dpp expression in leading edge cells.

**dppF11 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 dppF11 expression. We examined embryos lacking maternal and zygotic gene function derived from females bearing arm2 or nej3 GLC. The hypomorphic alleles arm2 and nej1 were used to make GLC because the null alleles arm1 and nej1 do not come through the germline (Peifer and Wieschaus 1990; Waltzer and Bienz 1998).

Weak dppF11 expression is seen at stage 12 in arm2 GLC embryos (Figure 4A). No lacZ expression is seen at later stages in arm2 GLC mutant embryos (Figure 4, C and E). dppF11 expression does not initiate during stage 12 in nej3 GLC mutant embryos (Figure 4B). Faint lacZ expression is seen at later stages in nej3 GLC mutant embryos (Figure 4, C and F). dppF11 expression in these embryos is likely due to the fact that arm2 and nej3 are not null alleles. In stage 17 embryos, each mutant’s effect on dppF11 expression matches the severity of its mutant phenotype. nej3 GLC and arm2 nej3 GLC 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.

**Med1 is a dominant enhancer of arm and nej effects on dppF11 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 dppF11 in arm1 or nej3 zygotic mutant embryos that were also heterozygous for Med1. We reasoned that if the two pathways were acting independently, then heterozygosity for Med1 (a recessive null allele) would have no effect on arm1 or nej3 regulation of dppF11 expression. However, if there were a synergistic interaction between the pathways, then the dosage of Med1 could influence the effect of arm1 or nej3 on the maintenance of dppF11 expression.

The initiation of lacZ expression from dppF11 in leading edge cells is largely unaffected in all embryos due to maternal contributions from each gene. However, dppF11 expression is well below wild-type levels in both Med1-enhanced zygotic mutant backgrounds at stage 17 (compare Figure 5, A and B, with Figure 1E). Of greater importance, the effect of arm1 or nej3 on dppF11 expression is more severe in the absence of one functional copy of Med1 than in their respective zygotic single mutants. To see the effect of heterozygosity for Med1, compare Figure 5A to Figure 3C for nej3 and compare Figure 5B to Figure 2B for arm1. Dominant enhancement of arm1 and nej3 zygotic mutant phenotypes by Med1 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 dppF11 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 requires 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 arm2 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 enhances dpp wing phenotypes, we show that Med enhances nej embryonic phenotypes. We extend their study by suggesting a role for nej in mediating combinatorial signaling by the Wg and Dpp pathways.

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/CREBBP 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 dppF11 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 \( \text{dpp} \) regulation in leading edge cells, our analysis of \( \text{dpp}^{F11} \) further establishes the value of the \( \text{hobo} \) genetic system as an analytical tool in Drosophila. Our study shows that (with the caveat that suitable strains must first be identified) the \( \text{hobo} \) 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 \( \text{hobo} \) genetic system that reflect its versatility. This study is the first to utilize plasmid rescue of sequences flanking \( \text{hobo} \) transgenes and the histochemical analysis of \( \beta \)-galactosidase expression from \( \text{hobo} \) enhancer trap vectors in embryos as analytical tools to address developmental questions. In addition, we describe a set of \( \text{hobo} \) sequencing primers for the analysis of rescued, flanking genomic DNA and the analysis of \( \beta \)-galactosidase expression from \( \text{hobo} \) enhancer traps in imaginal discs.

Like many genetic analyses, our study of \( \text{dpp}^{F11} \) was conducted over several years. This allows us to address important issues about the long-term stability of \( \text{hobo} \) 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 \( \text{hobo} \)-associated mutant. Regarding the stability of \( \text{hobo} \) transgenes in stocks and in crosses, we found absolutely no evidence of instability. In our hands, this issue is no more relevant for \( \text{hobo} \) than it is for \( P \). The \( \text{dpp}^{F11} \) 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 \( \text{dpp}^{F11} \) strain. For example, the strain always demonstrates haploinsufficiency when recombinant progeny with the \( \text{hobo} \) insertion but without the \( \text{dpp} \) 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 \( \text{hobo} \)-associated mutant, we admit that this is more tedious than using the \( P \) system. The trade-off is that \( P \) and \( \text{hobo} \) elements have distinct insertion preferences. This was shown in a genome-wide survey (Smith et al. 1993) and in an analysis at the \( \text{dpp} \) locus (Newfeld and Takaesu 1999). In addition, there is no \textit{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 \( \text{hobo} \) 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 \( \text{hobo} \) 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 \( \text{hobo} \) genetic system.

From a genome-wide perspective, the majority of predicted genes are not yet muagenized 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 \( \text{hobo} \) to extend the reach of current mutagenesis methods. It seems likely that the \( \text{hobo} \) 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 \( \text{hobo} \) 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 \( \text{dpp}^{F11} \) expression in leading edge cells by Dpp and Wg will likely have wide relevance to TGF-\( \beta \) and Wnt signaling in many species.

We thank Brian Calvi for \( \text{hobo} \) sequencing primers. We thank Esther Siegfried and Mike O’Connor for valuable discussions and Ann Bradley for help with fly stocks. Ray Marquez, Will Sewall, Omar Sultani, and Ross Waldrip assisted with lacZ staining and Aaron Johnson provided assistance with image analysis. We thank Mariann Bienz, Beth Noll, and the Bloomington Stock Center for strains. This research was supported by a Basil O’Connor Starter Scholar Research Award from the March of Dimes, a Research Incentive Award from Arizona State University, and a grant from the National Institutes of Health (CA-95875).

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Communicating editor: R. S. Hawley