Promoter specificity mediates the independent regulation of neighboring genes

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Although enhancers can exert their influence over great distances, their effect is generally limited to a single gene. To discern the mechanism by which this constraint can be mediated, we have studied three neighboring *Drosophila* genes: *decapentaplegic* (*dpp*), *SLY1* homologous (*Slh*) and *out at first* (*oaf*). Several *dpp* enhancers are positioned close to *Slh* and *oaf*, and yet these genes are unaffected by the *dpp* elements. However, when a transposon is located within the *oaf* gene, the *dpp* enhancers activate the more distant transposon promoters while still ignoring the closer *Slh* and *oaf* start sites. To test whether this promoter specificity accounts for the regulatory autonomy normally found for the three genes, we used in vivo gene targeting to replace the *oaf* promoter with a *dpp*-compatible one in an otherwise normal chromosome. Strikingly, this chimeric gene is now activated by the *dpp* enhancers. Thus, the properties of the promoters themselves are sufficient to mediate the autonomous regulation of genes in this region.

[Key Words: Promoter specificity; targeted gene replacement; *Drosophila*; promoter/enhancer interaction; gene regulation]

Received February 21, 1996; revised version accepted April 3, 1996.

Enhancers can function over great distances and interact with almost any kind of promoter. These two features underscore a fundamental concern of gene regulation: How is an enhancer constrained to activate only its appropriate gene while not affecting adjacent ones? With the burgeoning of human gene therapy, this issue of regulatory insulation has become one of practical significance. In particular, it is crucial that regulatory sequences introduced with the therapeutic transgene not affect the expression of the genes near its insertion and vice versa.

For the same reasons, each gene in the cell must maintain its intergenic regulatory autonomy (IRA). To date, experimental evidence has pointed to two distinct models that explain how IRA can be mediated. The first model proposes that aspects of chromatin structure serve to divide the chromosome into independent domains of gene action and that a given enhancer and promoter can interact only if they reside within the same domain (Kitsberg et al. 1991; Laemmli et al. 1992; Dillon and Grosfeld 1994; Lewin 1994). It is generally believed that boundary structures, stable nucleoprotein complexes, are formed at the ends of the domain and serve to establish and demarcate the functional unit (Eissenberg and

Elgin 1991; Kitsberg et al. 1991; Laemmli et al. 1992; Lewin 1994). Several sequences, which we term domain elements, exhibit properties suggesting that they can establish such boundaries. In vertebrates, these include the locus control region (LCR) of the globin gene cluster (Forrester et al. 1986; Grosfeld et al. 1987; Chung et al. 1993) and the A elements of the lysozyme gene (Stief et al. 1989; Bonifer et al. 1990; McKnight et al. 1992). In *Drosophila*, domain element characteristics are associated with the specialized chromatin structures *scs* and *scs'* of the *hsp70* locus (Kellum and Schedl 1991; Vasquez and Schedl 1994) and the *su(Hw)* binding region within the *gypsy* retrotransposon (Holdridge and Dorsett 1991; Geyer and Corces 1992; Roseman et al. 1993). Most of these domain elements can also act ectopically as insulators to prevent enhancers on one side from acting on promoters on the other side (Stief et al. 1989; Kellum and Schedl 1992; Chung et al. 1993; Cai and Levine 1995; Scott and Geyer 1995). This property is crucial for maintaining IRA.

The second model, termed promoter specificity, states that inherent properties of the promoters and enhancers themselves allow some combinations to interact while others are ineffectual. Such a lock-and-key mechanism is likely to be mediated by the compatibility of the proteins bound at the regulatory elements. Recent work on the ability of an enhancer to utilize alternative promoters differentially within the *Adh* gene has shown that the discrimination is mediated by differing TFIID-TFIIA

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complexes at the promoters (Hansen and Tjian 1995). To date, little is known regarding the use of this mechanism for mediating intergenic autonomy. Experiments involving transgenic constructs in *Drosophila* suggest that promoter specificity best explains the independence of the adjacent *gooseberry* and *gooseberry-neuro* genes (Li and Noll 1994), as well as some aspects of Antennapedia Complex expression (Gindhart et al. 1995; Kapoun and Kaufman 1995). However, these experiments were all done using approaches in which the gene's normal chromatin environment had been disrupted.

Our interests in IRA arose from the characterization of the *Drosophila decapentaplegic* (*dpp*) gene. Among its many roles, *dpp* is required for the normal development of imaginal disks (Spencer et al. 1982), the groups of cells destined to become the adult's appendages. Expression of *dpp* in the disks is controlled by a 30-kb *cis*-regulatory region, called the disk region, located at the 3' end of the gene (Fig. 1) (Masucci et al. 1990; St. Johnston et al. 1990; Blackman et al. 1991). We have identified numerous enhancers in this region, each controlling a distinct subset of the *dpp* expression pattern in the disks (Blackman et al. 1991; R. Blackman, unpubl.). These enhancers reside 20–35 kb from their preferred *dpp* promoter (Fig. 1).

The great distance over which the *dpp* enhancers exert their influence prompted us to examine whether these enhancers also affect other genes in their vicinity. Our initial search for genes near the disk region uncovered the *out at first* (*oaf*) gene (Bergstrom et al. 1995). In this paper we report that another gene, named *SLY1* homologous (*Slh*), lies between *dpp* and *oaf*. Both *oaf* and *Slh* are immediately adjacent to the disk region (Fig. 1) and yet they are unaffected by these *dpp* enhancers (described herein).

The proximity of *dpp*, *Slh*, and *oaf* suggests that mechanisms must be in place to limit the interaction of the *dpp* enhancers with the adjacent genes. Our ability to elucidate this mechanism has been greatly aided by the insertion of an enhancer trap transposon into the 5' end of the *oaf* gene. In this report we first examine the regulation of the promoters in and around the transposon and, second, we use the transposon to mediate in vivo gene targeting (Gloor et al. 1991; Nassif et al. 1994) to replace the *oaf* promoter with another. This technique has allowed us to examine the effect of the promoter

exchange in an otherwise normal chromosome. Using these approaches, we present definitive evidence for the key role of promoter specificity in mediating IRA in the *dpp*–*oaf* interval.

Results

A new gene near *dpp*

To identify functionally important sequences near the *dpp* enhancers, we used DNA sequence comparisons between two distantly related species, *Drosophila melanogaster* and *Drosophila virilis* (Blackman and Meselson 1986). This comparison uncovered an extensive region of homology, likely to be a new gene, in the 119–121.5 region of the *D. melanogaster* map. Taking into account three probable introns (each <80 nucleotides long), the *D. melanogaster* sequence (GenBank accession no. U46124) has the potential to encode a protein of 655 amino acids. A BLAST search (Altschul et al. 1990) of GenBank shows that this sequence is strongly related throughout its entire coding region to the *SLY1* gene of yeast, coding sequence F43D9.3 from *Caenorhabditis elegans*, and the *r-sly1* gene of rat with amino acid identities of 32%, 44%, and 55%, respectively. The rat and fly genes have 81% amino acid similarity. Because of this extensive similarity, we have named our gene *Slh*. Another *Drosophila* gene, *Rop*, also shows sequence similarity to *SLY1* (Salzberg et al. 1993), but it has only 18% identity and its regions of similarity do not encompass the entire coding region. The sequence identity between *Rop* and *Slh* is only 11%, suggesting that their functions have diverged greatly.

In yeast, *SLY1* is an essential gene involved in vesicle transport between the endoplasmic reticulum and the Golgi apparatus (Dascher et al. 1991; Ossig et al. 1991; Mizuta and Warner 1994). In flies, however, *Slh* appears to be a nonessential gene because a chromosome with a deletion for most of the *Slh*-coding region is homozygous viable (Bergstrom et al. 1995).

Although a detailed analysis of *Slh* is beyond the scope of this paper, certain aspects of its transcriptional regulation are pertinent. The sequence comparisons predicted that *Slh* RNAs should begin near the *oaf* gene and

Figure 1. The molecular organization of *dpp*, *Slh*, and *oaf*. The map coordinates (in kilobases) are based on a previous genomic walk (St. Johnston et al. 1990; Bergstrom et al. 1995) and are slightly adjusted as a result of more accurate mapping. For *Slh* and *oaf*, the transcription units, including introns, are shown as black arrows. The genes are divergently transcribed with only 300 bp

separating their start sites. For *dpp*, three alternative first exons (shown as rectangles) are connected by broken lines to the arrow representing exons 2 and 3 and the intervening intron, which are common to all *dpp* transcripts. Other minor start sites for *dpp* RNAs are not shown. Imaginal disk-specific expression of *dpp* is controlled by enhancers (solid ovals) located within the 3' disk regulatory region. These enhancers initiate transcription primarily at the start site depicted at map coordinate 79 (St. Johnston et al. 1990; R. Blackman, unpubl.).

be transcribed toward *dpp*. Having found *Slh* transcripts in embryos (see below), we used 5' rapid amplification of cDNA ends (RACE) (Frohman et al. 1988) on embryonic RNA to map the start of transcription for *Slh* to a site 300 bp upstream of *oaf* (see Materials and methods). Thus, *oaf* and *Slh* are divergently transcribed with only 0.3 kb separating their respective start sites (Fig. 1). The two promoters have no apparent sequence homology.

dpp, *Slh*, and *oaf* are independently regulated

To compare the expression pattern of *Slh* with *dpp* and *oaf*, we performed whole-mount in situ hybridization on embryos and larvae. In embryos, *Slh* RNA is first evident at stage 14, with signal in the posterior midgut, esophagus, and salivary glands, and remains in these tissues through the remainder of embryogenesis (data not shown). This expression pattern is distinct from that of *oaf* (Bergstrom et al. 1995) or *dpp* (St. Johnston and Gelbart 1987) and shows that these closely situated genes are independently regulated.

For this analysis, we used three different single-stranded probes covering the *Slh* region. Probes extending (5' → 3') from 119.5 to 121.0 (containing only *Slh* antisense sequences) or from 115.1 to 121.0 gave identical results (as described above), whereas a probe extending from 120.8 to 115.1 (containing the sense strand of *Slh* plus flanking sequences) did not detect expression in any embryonic tissue (data not shown). These results demonstrate that our probes are capable of discriminating localized *Slh* expression in tissues and suggest that no other embryonic gene is present in the 115–121 interval.

We also used these same probes to examine *Slh* expression in late third-instar larvae and found no evidence for *Slh* expression in the anterior structures of these animals. In particular, there was no accumulation of *Slh* transcripts in the imaginal disks (Fig. 2). At the same time, we also performed hybridizations with probes specific for *dpp* and *oaf* transcripts (Fig. 2). The *dpp* probe yielded its characteristic pattern of a stripe abutting the anterior–posterior compartment boundary in each of the disks (Masucci et al. 1990; Blackman et al. 1991; Raftery et al. 1991). Like *Slh*, the *oaf* probe failed to display any reproducible localized pattern within the disks. Unlike *Slh*, however, the *oaf* probe always gave a low level of uniform staining in all of the disks. We do not know whether this represents a low level of ubiquitous RNA accumulation or a higher background level specific to the *oaf* probe. In either case, it is clear that the *Slh* and *oaf* transcripts do not accumulate in a *dpp*-like pattern in the imaginal disks. Thus, the *dpp* enhancers do not activate the promoters of these nearby genes in the wild-type chromosome.

The dpp enhancers can activate heterologous promoters in their vicinity

Previously, we characterized (Bergstrom et al. 1995) an enhancer trap line, called E32 (Brook et al. 1993), whose P element is inserted within the transcribed, 5'-untrans-

Figure 2. RNA expression in wild-type imaginal disks. Whole-mount in situ hybridization with late third-instar disks was performed with riboprobes specific for *dpp*, *Slh*, or *oaf* transcripts. For *dpp* and *oaf*, cDNA clones were used to prepare the probes. For *Slh*, genomic DNA fragments were used (see text). Data shown in this and subsequent figures were generated from the *Slh* probe spanning the 119.5–121.0 interval. (Left) In the wing disk, *dpp* is expressed in a stripe abutting the A/P boundary in the ventral hinge (vh), wing pouch (wp), dorsal hinge (dh), and notal (n) regions and in the peripodial membrane (pm). The expression in the presumptive alar lobe (al) region is probably not under the control of the disk region enhancers (Blackman et al. 1991; R. Blackman, unpubl.). In the leg disk, *dpp* transcripts accumulate along the A/P boundary from the presumptive dorsal thoracic (dt) region through the end knob (ek, the presumptive tarsal segments) and into the ventral quadrant (vq). (In this and subsequent figures, mesothoracic leg disks only are shown. Pro- and metathoracic leg disks have identical patterns.) In the eye disk, *dpp* is expressed in the morphogenetic (mf) furrow and in the disk periphery extending from the furrow to the antennal disk. In the antennal disk, *dpp* RNA accumulates in a stripe passing through the presumptive arista (a). (Center) *Slh* is not expressed in the imaginal disks. (Right) The *oaf* probe does not identify any reproducible localized pattern in the disks but instead binds at a low level throughout. We have not determined whether this represents a low level of ubiquitous *oaf* RNA accumulation or a higher background level specific to this probe.

lated region (UTR) of *oaf* (Fig. 3). The transposon, inserted after nucleotide +40, is oriented such that its *lacZ* reporter gene and *oaf* are transcribed in the same

Figure 3. Expression patterns in the *E32* line. (A) The expression patterns (from left to right) of *dpp*, *Slh*, *lacZ*, and *oaf* in flies homozygous for the *E32* transposon. The pattern of *dpp* RNA is indistinguishable from that of wild type, suggesting that *dpp* expression is not influenced by the insertion of the transposon. No expression is observed for *Slh*, showing that its pattern is also unchanged from wild type. Histochemical staining for β -gal (the product of the *lacZ* gene) shows that this gene's expression is under the regulatory influence of the *dpp* enhancers. In the wing disk epithelium, the staining reproduces much of the *dpp* pattern along the A/P boundary. Unlike *dpp*, no staining is observed in the upper notal or dorsal hinge regions. The leg disk has expression in the dorsal thoracic and end knob regions with a few clusters of cells staining more intensely. No expression is observed in the ventral quadrant of the disk. In the antennal disk, β -gal is present in the cells fated to become the arista and antenna segments. There is no staining in the eye disk. The expression pattern of *oaf* RNA is the same as that of the *lacZ* gene, showing that its expression in the disks is also controlled by the *dpp* enhancers. (B) Schematic representation of the chromosome carrying the *E32* insertion. The thick arrows indicate the direction and extent of the *Slh* and *oaf* transcription units. The *E32* transposon (shaded) is inserted after nucleotide +40 of the *oaf* gene. The darker shaded regions of the transposon correspond to the *lacZ* reporter gene and the *ry*⁺ gene, whereas the regions marked P represent the P-element terminal sequences. The bent arrows indicate the sites of the promoters within the transposon that are activated by the *dpp* disk enhancers (solid ovals). *lacZ* transcripts begin around nucleotide +67 of the P element, whereas *oaf* RNAs begin at a promoter within the right end of the transposon. No transcripts in the disks begin at the endogenous *Slh* or *oaf* promoters.

direction, with their respective promoters ~ 100 bp apart. The 15-kb transposon also contains the *Drosophila rosy*⁺ (*ry*⁺) gene (used as an eye color marker for transformant selection). Whereas null *oaf*[−] mutations are ordinarily lethal (Littleton and Bellen 1994; Bergstrom et al. 1995), flies homozygous for the *E32* insertion are phenotypically normal and can be maintained as a stock. Functional *oaf* transcripts in the *E32* line are initiated at a cryptic promoter lying near, but within, the right end of the transposon and continue through the *oaf*-coding region (Fig. 3B; Bergstrom et al. 1995).

Histochemical staining shows that the β -galactosidase activity (β -gal) pattern observed in the *E32* line resembles, in general, the normal *oaf* expression pattern (Bergstrom et al. 1995). There is, however, one critical difference. In *E32* larvae, we observe β -gal in stripes in each of the imaginal disks (Fig. 3), whereas in wild-type larvae there is no such *oaf* RNA pattern in these tissues (Fig. 2). In the wing disk epithelium, β -gal is expressed along the anterior–posterior (A/P) compartment boundary in the cells fated to become the wing, notum, and the ventral wing hinge tissues. In the leg disks, the stripe extends from the presumptive dorsal thoracic region to the central end knob (the presumptive tarsal segments) with several areas staining more strongly. In the eye/antennal disk, only the presumptive arista region produces β -gal. These patterns constitute a substantial subset of the normal *dpp* RNA pattern and approximate the pattern directed by the *dpp* disk enhancers lying in the 110–117 interval (R. Blackman, unpubl.). It is striking that the transposon should respond to the *dpp* disk enhancers, whereas the *Slh* and *oaf* promoters, located in nearly the identical position in the wild-type chromosome, do not.

These results suggest that the *dpp* enhancers preferentially interact with specific promoters. These enhancers do not activate the promoters of the adjoining genes but can activate a compatible promoter inserted beyond them. An alternative explanation, however, is that the novel action of the *dpp* enhancers in the *E32* chromosome results from an altered chromatin environment. In this view, the insertion of the *E32* transposon may disrupt a chromatin-based mechanism that separates the *dpp* and *Slh/oaf* genes into separate domains. This perturbation would then allow the *dpp* enhancers to inappropriately activate any promoter in their vicinity.

To resolve this issue, we have examined more closely the transcription of the *dpp*, *Slh*, *oaf*, and *lacZ* genes in the *E32* line. If the transposon does disrupt the domain structure separating *dpp* from the other genes, then we would expect that each of the genes would interact with the disk enhancers and be transcribed in a *dpp*-like pattern. If promoter specificity mediates IRA in this interval, however, only the ectopic *lacZ* promoter should be activated, whereas the *Slh* and *oaf* promoters will remain quiescent.

The expression pattern of Slh and oaf in the E32 line supports the promoter specificity model

We first determined the effect of the transposon on the

expression patterns of the genes in the region (Fig. 3). In situ hybridization for *dpp* transcripts showed that its expression is normal. Likewise, the pattern of *Slh* was unaffected by the transposon, showing no expression in the disks. Hybridizations with an *oaf* cDNA probe, however, uncovered a new pattern. In *E32*, *oaf* RNA is present in a pattern virtually identical to that seen for the β -gal staining. These transcripts are clearly driven by the *dpp* enhancers and presumably initiate at the cryptic promoter within the right end of the transposon.

We also examined whether the *oaf* start site or a P-element promoter is used to initiate the *lacZ* transcripts. In enhancer trap lines such as *E32*, it is assumed that these RNAs initiate at the promoter of the P-element transposase gene. Two different start sites, at +67 and +87, have been found for transposase transcripts (Karess and Rubin 1984; R. Blackman, unpubl.). Using 5' RACE on larval RNA from the *E32* line, we found that essentially all transcripts in these animals initiate at or near the +67 start site within the P element (see Materials and methods). Importantly, none of the RACE products began at the normal *oaf* promoter.

Thus, as diagrammed in Figure 3B, these results indicate that the *dpp* enhancers ignore the nearer *Slh* and *oaf* promoters while activating two transposon promoters, one at essentially the same location and the other 15 kb away. This use of only some promoters in the region strongly supports the notion that promoter specificity is the overriding feature establishing the regulatory autonomy of these genes.

A direct test of the promoter specificity model

To test definitively whether promoter specificity is mediating IRA in this interval, we needed to show that the inability of the *dpp* enhancers to activate *Slh* and *oaf* is mediated exclusively through the promoters of these

genes. Ideally, these assays should be done with the genes in their normal chromatin environment. We accomplished both of these objectives by replacing the endogenous *oaf* promoter with a compatible one using P element-mediated gene replacement (Nassif et al. 1994). This method makes use of the fact that a gap in the chromosome is created when a P element excises. To repair the gap, the cell normally uses the homologous chromosome as the template to replace the missing information (Engels et al. 1990). The Engels and Gloor laboratories have demonstrated, however, that homologous, but altered, sequences located at an ectopic site can also serve as the template (Gloor et al. 1991; Nassif et al. 1994). Thus, using this approach, we could replace the sequences near the *E32* insertion site with any that we desired. An important feature of this approach is that the transposon is lost during the process, and the changes are introduced into an otherwise normal chromosome.

We created the replacement template P{ySWAP} by substituting the -52 to +47 interval of the *oaf* promoter with the -50 to +65 region of an *hsp70* promoter. Because removal of the *oaf* sequences required the creation of two restriction sites, three additional nucleotide changes were introduced into the replacement template (Fig. 4). We selected this *hsp70* promoter, which lacks the heat shock response element, because it had been activated by the *dpp* enhancers in other constructs (Blackman et al. 1991; R. Blackman et al., unpubl.). To provide sufficient homology for targeting, the replaced promoter was embedded within a 4.8-kb fragment from the *oaf* region.

To produce the targeted replacement, we generated flies possessing the ectopic P{ySWAP} template, the *E32* transposon, and the transposase-producing P{ Δ 2-3} element (see Materials and methods). To reduce the chance that the homologous chromosome would be used for repair, we remobilized *E32* over a rearranged second chromosome that disrupts pairing in the region near *oaf*

Figure 4. Comparison of the *oaf* and *oaf*^{SWAP} promoters. The nucleotide numbers given are relative to the start of *oaf* transcription except for those in the shaded boxes, which are distances relative to the *hsp70* start site. In the *oaf*^{SWAP} chromosome, the -52 to +47 sequence of the normal chromosome has been replaced with the *hsp70* promoter (shaded). Three base pair changes (solid ovals), used to create restriction sites needed for the replacement, are also present in the *oaf*^{SWAP} chromosome. The two chromosomes differ in length by 16 bp, and the start of *oaf*^{SWAP} transcription is only 2 bases closer to the *dpp* enhancers than is the normal *oaf* start site. The start of translation for *oaf*^{SWAP} is now 93 bp downstream of the start of transcription. The TATA box of the *oaf*^{SWAP} sequence is marked; the *oaf* promoter lacks this motif. Each promoter contains a near-consensus Initiator (Inr) sequence (Cherbas and Cherbas 1993) at the start site. The sequence of the *oaf* promoter from -60 to +55 is 5'-dGTCACACCTGT-CAGCTGATTTTCGAAAAGTCACGTTTCAGTCAGTTTCACCATTTTCTGCCAGTTATTTTTCGTTTCGTCGACGCGAGTGGT-TCGTTGTGCTTTCTTGTGATTTTAT. The corresponding *oaf*^{SWAP} sequence is 5'-dGTCAGAtCCGAAAAGAGCGCCGGAG-TATAAATAGAGGCGCTTCGTCTACGGAGCGACAATTCAATTCAAACAAGCAAAGTGAACACGTCGCTAAGCGAAAGCT-AAGCAAATAAACAAGCGCAGGATcTTAT. Nucleotide changes resulting from the introduced restriction sites are noted by lowercase letters and the *hsp70* sequence is underlined.

(Gelbart 1982). Flies were selected for loss of the *ry*⁺ gene function associated with the *E32* transposon. This phenotype indicates remobilization of the transposon and potential replacement of the *oaf* promoter. The offspring of 112 *ry*⁻ flies were screened by PCR for the precise replacement of the *oaf* promoter with the *hsp70* sequences. Of these lines, one contained a precise replacement that was verified by both PCR and sequencing. We will refer to this altered gene as *oaf*^{SWAP}. Homozygous *oaf*^{SWAP} flies appear wild type and can be maintained as a robust stock.

To determine how the altered promoter would affect transcription of the genes in the region, we performed *in situ* hybridization using *dpp*, *Slh*, and *oaf* probes on imaginal disks from homozygous *oaf*^{SWAP} third-instar larvae (Fig. 5). *dpp* and *Slh* are expressed in their normal patterns, demonstrating that they are unaffected by the altered *oaf* gene. In contrast, *oaf*^{SWAP} RNA accumulates in a *dpp*-like expression pattern. *oaf*^{SWAP} expression extends along the A/P boundary of the wing disk in the hinge, wing pouch, and notal regions. It differs from the *dpp* pattern in lacking a portion of the notal expression. In the leg, *oaf*^{SWAP} expression appears along the A/P boundary extending from the dorsal thoracic region to the end knob of the disk. Unlike *dpp*, the expression of *oaf*^{SWAP} does not continue from the end knob to the periphery on the ventral side of the disk. In the eye/antennal disk, *oaf*^{SWAP} accurately reproduces most of the *dpp* pattern, including the expression in the morphogenetic furrow. As judged by the time needed to produce the signals shown in Figure 5, the levels of *dpp* and *oaf*^{SWAP} expression are fairly comparable. These results show clearly that *oaf*^{SWAP} responds effectively to the *dpp* enhancers to activate transcription in the disks.

Discussion

Promoter specificity, not domain boundaries, mediates IRA in this interval

Our results demonstrate conclusively that promoter elements are an essential feature of the mechanism maintaining the regulatory autonomy of the genes within the *dpp*–*Slh*–*oaf* interval. First, in the *E32* line, we show that the *dpp* enhancers ignore the nearer *Slh* and *oaf* promoters but still activate two more distant P-element promoters. This finding provides strong support for promoter specificity and adds another important example to the list of genes for which this mechanism is thought to maintain IRA (Li and Noll 1994; Gindhart et al. 1995; Kapoun and Kaufman 1995). The results from each of these studies, however, were obtained under conditions where the assayed promoters and enhancers were not in their native chromatin environments. Thus, any additional aspects of IRA mediated through chromatin structure could not be analyzed in these experiments.

To address this issue, we have tested our model in a completely normal chromatin context. Using a P-element-mediated method for substituting sequences *in vivo* (Nassif et al. 1994), we replaced the *oaf* promoter

Figure 5. RNA expression patterns in disks from homozygous *oaf*^{SWAP} larvae. Whole-mount *in situ* hybridization was performed with riboprobes specific for *dpp*, *Slh*, or *oaf* RNA. (Left) The promoter replacement does not affect transcription of the *dpp* gene. Its expression is indistinguishable from wild type. (Center) Expression of *Slh* RNA is also not affected by the replacement and shows no localized accumulation. (Right) With its *hsp70* promoter, *oaf*^{SWAP} is activated by the disk enhancers and reproduces nearly all of the *dpp* pattern. In the wing disk, *oaf*^{SWAP} RNA appears along most of the A/P boundary and differs from *dpp* only in lacking staining in part of the notal region. In the leg disk, the entire dorsal thoracic and end knob pattern is seen but there is no reproducible staining in the ventral quadrant. In the antennal disk, *oaf*^{SWAP} RNA is present in the cells fated to become the arista and antenna. Finally, in the eye disk, *oaf*^{SWAP} transcripts accumulate in a discrete stripe in the morphogenetic furrow, exactly the same as *dpp*. Interestingly, this implies that the *oaf*^{SWAP} RNA turns over in the furrow with the same kinetics as the *dpp* transcripts even though these RNAs have no sequence homology.

with one that is compatible with the *dpp* enhancers, in effect altering only 100 bp of the chromosome. As predicted by the promoter specificity model, the chimeric *oaf*^{SWAP} gene responds to the disk region enhancers and is expressed in a pattern essentially identical to that of *dpp* in the disks. This clearly demonstrates that inherent properties of the promoter are sufficient to determine IRA.

Equally important, our data rule out the presence of an IRA-establishing domain boundary anywhere within the *dpp*–*Slh*–*oaf* interval. Thus, domain boundary-mediated IRA, although important for some genes, may not be as

widespread as suggested previously (Corces 1995). For the reasons described below, we feel that promoter specificity may be a more common mechanism for IRA.

Domains as mediators of IRA

There is ample biochemical and cytological evidence that active genes are found within domains of chromatin whose structure is distinct from that of quiescent genes (Elgin 1990; Lewin 1994). It is still unclear, however, whether these biochemically defined units always have a functional role in mediating IRA (Dillon and Grosveld 1994; Schedl and Grosveld 1995). In the reported cases where domain elements establish effectual domains [the globin, lysozyme, heat shock, immunoglobulin, and Bithorax Complex genes (Schedl and Grosveld 1995)], it is interesting to note that nearly all are associated with abundantly expressed loci. It is quite possible that insulated domains are needed principally for cases of exceptionally active genes.

The mechanism by which these domain elements function is unknown and is complicated by the fact that several of the elements have additional regulatory functions. In particular, the globin and lysozyme LCRs contain several gene-specific enhancers (Grosveld et al. 1987; Stief et al. 1989), whereas the *scs'* element of the heat shock locus contains the promoters for two divergently transcribed, non-heat shock genes (Glover et al. 1995). Further experimentation is needed to discern whether all these domain elements function in similar or distinct manners to mediate IRA.

Given the vast gene number and regulatory complexity of the eukaryotic genome, it is likely that all of the above mechanisms, and more, are needed by the cell to maintain IRA. The list of characterized genes is still too small to know whether any one mechanism will predominate.

Promoter specificity defined

Implicit in our proposal for promoter specificity is the premise that the *dpp* enhancers ignore the promoters of the adjacent genes. Our experiments are unable to distinguish whether this results from the physical inability of the enhancers to interact with the promoters or from the active repression of the promoters by gene-specific negative regulatory elements. Although we favor the former explanation, the *oaf*^{SWAP} results demonstrate that regardless of the mechanism, the inactivity of the *oaf* gene is dependent on the identity of its promoter. For this reason, we have adopted the term promoter specificity to describe such cases where an enhancer has the inherent ability to interact with some, but not all, promoters in its vicinity. We contrast this mechanism with promoter selectivity, in which an enhancer is capable of activating multiple promoters but opts to activate only one at a given time. This is typically observed in intragenic regulation, where an enhancer selects between alternative promoters of a gene in different tissues or developmental stages [e.g., the *Drosophila Adh* and *actin* genes (Corbin and Maniatis 1989; Chung and Keller

1991; Hansen and Tjian 1995)], but it has also been observed in intergenic situations where an enhancer is shared by separate genes [e.g., the globin loci (Choi and Engel 1988)].

Mechanisms mediating promoter specificity

It is generally assumed that promoter specificity (and selectivity) is mediated by the presence or absence of distinct protein complexes at the promoter and enhancer elements. This mechanism would require the cell to have many functionally different protein complexes capable of binding to promoters and initiating transcription. Given the large number of tissue-specific DNA-binding proteins (Wingender et al. 1996), the varying compositions of the general transcription factor complexes (Parvin et al. 1992; Martinez et al. 1994; Verrijzer et al. 1995), and the heterogeneity of the sequences surrounding promoters (Arkhipova 1995), it seems reasonable that such an extensive array of initiation complexes exists. The important functional differences between promoter sequences can be quite subtle (Das et al. 1995). An example of this was demonstrated by experiments analyzing the TATA elements of the myoglobin and SV40 early gene promoters (Wefald et al. 1990). Although SV40 has the sequence TATTTAT, the myoglobin sequence is TATAAAA. Replacement of the myoglobin TATA with that of SV40 abolished the ability of the myoglobin promoter to respond to a muscle-specific enhancer.

Equally subtle sequence characteristics are probably also responsible for the ability of a promoter to be activated by the *dpp* disk region enhancers. From our analysis, we know of three compatible promoters: the *dpp* promoter at map position 79, which initiates nearly all *dpp* transcription in the imaginal disks (St. Johnston et al. 1990; R. Blackman, unpubl.), the *oaf*^{SWAP} promoter (mostly that of *hsp70*), and the P-element +67 promoter. Four promoters are not activated by the enhancers: the *Slh* and *oaf* promoters, the P-element +87 promoter, and the *dpp* promoter at map position 83. Of these, the *oaf*^{SWAP} and P +87 promoters contain TATA boxes, whereas all but *Slh* and P +87 contain near-consensus Initiator (Inr) elements (Cherbas and Cherbas 1993) at their start sites. The presence of these common promoter motifs in both compatible and noncompatible promoters indicates that neither alone is sufficient to mediate the promoter specificity of the *dpp* enhancers. Using sequence comparisons, we have been unable to discern any motif that distinguishes between the two classes of promoters.

An alternative mechanism by which promoter specificity could be mediated is one in which the promoter establishes a local chromatin structure that is amenable to certain enhancers but not others. This perturbation (e.g., DNA bending) would have to be quite small in scale, as our experiments showed that the *dpp* enhancers are capable of differentiating between promoters separated by <300 bp in the *oaf*^{SWAP} chromosome and 110 bp in the *E32* chromosome. These distances are less than

that occupied by two nucleosomes and attest to the local nature of the promoter's effect.

An enhancer can equally activate one or more promoters

Although our results demonstrate the ability of *dpp* enhancers to remain indifferent to the *Slh* and *oaf* promoters, this does not preclude their ability to activate other promoters in the genome. Presumably, other aspects of physical distance or chromosome structure serve to limit their ability to interact with these distantly located, compatible promoters. When such promoters are brought nearby, however, as in the case of the *E32* or *oaf^{SWAP}* lines, the *dpp* enhancers show their flexibility by equally activating multiple promoters in the same cell. Strikingly, the presence of the ectopic promoters does not appear to lessen the level of *dpp* expression in these cells. Although in situ hybridization provides limited quantitative information, our data indicate that the disk region enhancers activate all three promoters in *E32* and both promoters in *oaf^{SWAP}* to a level similar to that of the *dpp* gene in a normal chromosome. Also, the extreme sensitivity of developing cells to concentrations of *dpp* protein (Ferguson and Anderson 1992; Wharton et al. 1993; Ingham and Fietz 1995; Zecca et al. 1995) serves as another metric to show that *dpp* expression has not been altered significantly. If the heterologous promoters had competed away a portion of the *dpp* enhancer activity, we should have observed *dpp*-like defects. Instead, homozygous *E32* and *oaf^{SWAP}* flies have normal phenotypes. It remains to be seen whether the *dpp* enhancers function to activate all of the promoters simultaneously or each one alternately, as observed for the LCR activation of human globin genes in transgenic mice (Wijgerde et al. 1995).

Promoter promiscuity

As our work demonstrates, the fact that an enhancer activates several promoters does not mean that it will interact with all promoters. Some promoters, though, appear to be more promiscuous than others. In the present study, the *hsp70* promoter was used because we had shown previously that it could be activated by the *dpp* disk enhancers (Blackman et al. 1991; R. Blackman, unpubl.). A search of FlyBase reveals that other investigators have used this sequence as a compatible promoter in their constructs as well. Because IRA of the *hsp70* locus is maintained by insulating domain boundaries (Kellum and Schedl 1991; Kellum and Schedl 1992; Vasquez and Schedl 1994) and not promoter specificity, it is possible that this promoter needs only to mediate efficient initiation without concern for intergenic enhancer interactions. Although the normal *hsp70* promoter maintains a paused RNA polymerase downstream of its start site (Lis and Wu 1993), the small portion of the promoter that we used in *oaf^{SWAP}* lacks critical sequences required for generating the paused complex (Lee et al. 1992). Thus, the paused polymerase is unlikely to account for the promoter's facility to be activated.

Most *Drosophila* enhancer trap transposons make use of the promiscuous nature of the P-element promoters to initiate expression of their reporter gene. Although this has proven very successful, estimates claim that >50% of the transposon insertions fail to report all of the regulatory information in their vicinity (Kania et al. 1995). It is likely that some of these failures are caused by the incompatibility of the enhancers with the P-element promoters. Future enhancer trap vectors, using other promiscuous promoters, may identify new genes that to date have evaded detection by the present class of transposons.

In vivo alterations of the Drosophila genome

We have made use of P-element-mediated targeted gene replacement (Nassif et al. 1994) to introduce our specific modifications into an otherwise normal chromosome. This represents the first such application of the technique for an autosomal location. In our experiments we could not predict a priori that the *oaf^{SWAP}* chromosome would survive over a deficiency; therefore, we remobilized the transposon over a rearranged chromosome thought to disrupt homologous pairing. Our ability to obtain the targeted replacement in the presence of a wild-type copy of the gene means that essentially any gene with a transposon insertion in or near it is amenable to sequence-specific alteration. With this added flexibility and the large pool of available transposon insertions, P element-mediated sequence replacement should become an indispensable tool for *Drosophila* molecular genetics.

Materials and methods

Fly strains

Unless noted, stocks used in this study are described in Lindsley and Zimm (1992). The *E32* enhancer trap line (Brook et al. 1993) contains the P{*lacZ^{PZ P T} kanR ori ry⁺t^{7.2}*=PZ} element inserted after nucleotide +40 of the *oaf* gene transcription unit (Bergstrom et al. 1995).

Sequence comparisons

DNA sequencing of *D. melanogaster* and *D. virilis* genomic DNA fragments was performed by the Genetic Engineering Facility at the University of Illinois. Sequences upstream of the *oaf* gene from both species were compared by use of the MacVector program. A 2.7-kb region of homology was identified and the conceptual translation product compared to the National Center for Biotechnology Information (NCBI) data bases by use of the BLAST program (Altschul et al. 1990).

5'-End determination of the Slh and E32 transcripts

The following oligonucleotide primers were used: S1, 5'-dACGCAAGGGCAGTTAGGTCACAAC-3'; S2, 5'-dGAAGTCATCTCCAGACTGA-3'; S3, 5'-dGTGCGTGGCCAGGCAGAA-GT3'; AP20, 5'-dGGCCACGCGTCGACTAGTAC-3'; L1, 5'-dGCTGCAAGGCGATTAAGTTG-3'; L2, 5'-dCAUCAUCAUCAUTGGGTAACGCCAGGGTTTTC-3'; P1, 5'-dGTGGGTATCACAAATTTGGG-3'; and P2, 5'-dGGTTCAGGCTCTATCACTTTACT-3'.

The 5' RACE System from Life Technologies was used to identify the start sites of the transcripts. To map the *Slh* 5' end, we started with RNA from 8- to 12-hr embryos. cDNA was prepared from 1 µg of total RNA with primer S1, located near the middle of the *Slh* transcription unit. One-quarter of the cDNA was poly(dC)-tailed and subjected to PCR in a 25-µl volume containing PCR buffer III (Ponce and Micol 1992), 200 µM dNTP, 1 unit of *Taq* polymerase (GIBCO/BRL), and 0.4 µM each of the Anchor Primer supplied with the kit and nested *Slh* primer S2. Samples were subjected to a thermocycling scheme of 3 min at 94°C; 25 cycles of 30 sec at 94°C, 30 sec at 54°C, and 30 sec at 72°C; and 10 min at 72°C. For the second round of PCR, we used primers S3 and AP20. The former is from *Slh* and lies nearer the 5' end of the gene, whereas the latter contains the 20 core nucleotides from the Anchor Primer. These primers were used in a 100-µl reaction containing 4 µl of round 1 product. The same PCR conditions were used as before except that 30 cycles were performed. This reaction produced a single product (when analyzed by agarose gel electrophoresis) of ~400 bp. The DNA was cloned into the pGEM-T vector (Promega), and eight clones sequenced. Seven terminated at the same nucleotide, and one was a single nucleotide shorter.

To map the 5' end of the transcripts responsible for the β-galactosidase expression in the *E32* line, we used RNA from wandering third-instar larvae. cDNA was primed with random hexamers, tailed, and used in PCR with the Anchor Primer and *lacZ* primer L1. Cycling conditions were 2 min at 94°C; 30 cycles of 1 min at 94°C, 1 min at 57°C, and 2 min at 72°C; and 10 min at 72°C. In controls with plasmid DNA or untailored cDNA, we found that the Anchor Primer inappropriately anneals to a sequence around nucleotide 300 of the P element, resulting in an artifactual product that was observed in later rounds of PCR amplification. From the first reaction, 0.05 µl of unpurified product was amplified with nested *lacZ* primer L2 and the Universal Amplification Primer supplied with the RACE kit. An appropriately sized fragment along with three smaller products were visible by gel analysis. A third round of PCR was done with the AP20 primer and either of two primers, P1 and P2, whose 5' ends are at nucleotide 293 and 379, respectively, of the P element. Gel analysis showed a single product in the reaction with P1 (260 bp) and two fragments with P2 (370 and 110 bp). In the latter reaction, the smaller fragment was the product resulting from the Anchor Primer inappropriately priming within the P element. The two largest fragments were cloned and sequenced. Of 23 clones, 21 ended at nucleotide 69 or 70 of the P element. The other two clones also ended within the P element. Similarly sized products were observed in other independently run experiments with different RNA preparations.

Expression patterns in imaginal disks

Whole-mount RNA in situ hybridization and histochemical staining for β-galactosidase activity were performed as described in Bergstrom et al. (1995). For these experiments, *y w* animals served as our wild-type control strain. All RNA probes were generated from DNAs subcloned into pBS II KS. Plasmid pBS-dppB1 contains a 3.2-kb *dpp* transcript B cDNA originally present in clone KP1 (St. Johnston et al. 1990). Plasmid pBS-oafNN9 contains a full-length 3.2-kb *oaf* cDNA from clone NN (Bergstrom et al. 1995). A 1.5-kb antisense riboprobe specific for *Slh* was prepared from genomic clone pBS119BH, covering the 119.5–124.4 interval, which had been linearized at an *Xho*I site at 121.0. Riboprobes covering the entire insert of clone pBS115CX, spanning the 115.1–121.0 interval, were prepared from each strand.

Construction of the P{ySWAP} template

To create the replacement template, we first subcloned a 1.9-kb *Xho*I–*Eco*RI fragment (map coordinates 120.8–122.7) containing the *oaf* promoter into the pALTER-1 vector from the Altered Sites II in vitro Mutagenesis System (Promega). Following the manufacturer's protocol, we created *Bgl*III sites at –57 to –52 and +47 to +52 relative to the start of *oaf* transcription. The resulting plasmid was cut with *Bgl*III, and the ends filled in with Klenow. The eliminated –52 to +47 sequence was replaced with the –50 to +65 sequence from the *hsp70* promoter found in plasmid pHSREM1 (Knipple and Marsella-Herrick 1988). The 1.9-kb *Xho*I–*Eco*RI fragment of this chimeric construct was used to replace its wild-type counterpart in a larger subclone extending 2.3 kb upstream and 2.8 kb downstream of the *oaf* start site. A 4.8-kb *Xba*I fragment from this plasmid was inserted into the *Xba*I site of the transformation vector P{y⁺ mDint = YC4} to create the replacement template P{y⁺ mDint oaf^{hsp70} P-SWAP = ySWAP}. P{YC4} (kindly provided by Pamela Geyer, University of Iowa, Iowa City) contains the intronless *yellow* (*y*) gene subcloned into the Carnegie 4 vector. P{YC4} transformants in a *y* background have wild-type body and wing pigmentation (Geyer and Corces 1987).

Gene targeting and promoter replacement

We injected P{ySWAP} and helper transposase DNA into *y ac; ry⁵⁰⁶* embryos using standard methods and recovered a transgene insertion, P{ySWAP}, on the X chromosome. Females of the genotype *y ac P{ySWAP}/y ac; E32/CyO; ry* were then crossed to *In(2LR)DTD42, dpp^{d-ho}; ry P{ry⁺ Δ2-3} 99B* males to induce excision of the *E32* transposon. *In(2LR)DTD42* is a rearranged second chromosome that is thought to disrupt chromosome pairing near *dpp* (Gelbart 1982), whereas the Δ2-3 P element serves as an immobile source of P transposase (Robertson et al. 1988). *y ac; E32/In(2LR)DTD42, dpp^{d-ho}; ry/ry P{ry⁺ Δ2-3} 99B* males were crossed individually to *dpp^{d-ho}; ry* females. Phenotypically *y⁻ dpp⁺ ry⁻* males of the genotype *y ac; E32^{*}/dpp^{d-ho}; ry* (where *E32^{*}* denotes the remobilized transposon chromosome) were recovered from 112 of the crosses. The loss of the *ry⁺* function from the *E32* chromosome signals that a remobilization event occurred, possibly leading to the gene-targeted replacement of the *oaf* promoter. The selected males were crossed to establish stocks. These were tested for the promoter replacement by PCR with two different primers inside the *hsp70* sequence paired with three different *oaf* gene primers. The *oaf* primers were from gene sequences not present in P{ySWAP} to ensure that proper PCR products would be amplified only from flies carrying the *hsp70* promoter in the native *oaf* gene. Of the 112 lines, one produced the appropriate PCR products expected of a replacement event. The amplified products were cloned and sequenced to confirm the precise promoter exchange. The chromosome bearing the chimeric gene, which we call *oaf^{SWAP}*, can be maintained as a homozygous stock and has wild-type phenotype.

Acknowledgments

We are grateful to Pedro Alvarez-Ortiz, Richard Shelby and Simi Thomas for technical assistance, Paige Goodlove of the University of Illinois Biotechnology Center for assistance in sequencing and primer design, and Sara Browning and Ellen Omi for help with maintaining the laboratory. We thank Pamela Geyer for supplying fly lines and the YC4 plasmid, Bill Engels for P-element primers and ideas, and Greg Gloor for his advice on the targeted gene replacement protocol. Also, we thank Pamela Geyer, Andy Belmont, and David Rivier for their insightful dis-

cussions and comments on the manuscript. Finally, R.K.B. especially expresses his gratitude to Joel Eissenberg for his perspicacity, eloquence, and antipodal nature—all of these have made this work and our friendship that much better. This work has been supported by grants to R.K.B. from the National Science Foundation (DCB 90-18618 and MCB 93-17701) and the University of Illinois Research Board. C.M. and D.E.B. were partially funded by National Institutes of Health predoctoral training fellowships.

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Note added in proof

Using RT-PCR, we have determined that *oaf* RNA is present in wild-type imaginal disks. Coupled with our *in situ* hybridization results, this suggests that the *oaf* promoter is competent for transcription in all disk cells. Thus, the failure of *oaf* to be activated by the *dpp* enhancers does not result from the inability of the promoter to function in these cells.

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C Merli, D E Bergstrom, J A Cygan, et al.

Genes Dev. 1996, **10**:

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