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Indirect autoregulation of a homeotic *Drosophila* gene mediated by extracellular signaling

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ABSTRACT **Commitments to developmental pathways are often made and maintained in groups of cells. Such commitments are conferred by the products of selector genes, many of which are homeobox genes. Homeobox genes can maintain their expression by directly autoregulating their own transcription. Here, we report a case where positive autoregulation of Ultrabithorax, a homeotic *Drosophila* gene, is at least partly indirect and mediated by the extracellular signal molecules that are products of the genes wingless and decapentaplegic. Indirect autoregulatory mechanisms may be used to ensure coordinate maintenance of selector gene activity in groups of cells.**

Selector genes (1), many of which are homeobox genes (2–5), are required until very late stages of development (6). Their activity must, therefore, be maintained throughout development, and positive autoregulation may be the mechanism used (7). Indeed, there are growing numbers of examples of selector genes (8–12) or other homeobox genes (13–15) that autoregulate their own expression. Evidence strongly suggests (16–18) that autoregulation in some of these cases is direct, i.e., mediated by the homedomain protein acting directly through regulatory sequences within its own gene. Interestingly, autoregulation can be dependent on extracellular signaling from neighboring cells (10, 12), suggesting that the process of autoregulation is not always entirely cell-autonomous.

Ultrabithorax (*Ubx*) is a *Drosophila* selector gene (19) whose expression in the embryonic visceral mesoderm requires Ubx function (8). Therefore, *Ubx* autoregulates its own expression in this germ layer and activates expression of the gene decapentaplegic (*dpp*) in the same cells (20–22). *dpp* function in turn is needed for expression of the gene wingless (*wg*) in adjacent cells (20). Thus, in the visceral mesoderm, *dpp* and *wg* are both target genes of *Ubx*:*dpp* may be a direct target gene, whereas *wg* is an indirect target gene (Fig. 1). *dpp* and *wg* encode extracellular proteins whose counterparts in mammals are transforming growth factor β and Wnt-1, respectively (23–25). Finally, all three genes (*Ubx*, *dpp*, and *wg*) are also target genes of abdominal A (*abd-A*), the homeotic gene expressed posteriorly adjacent to *Ubx* (26): *wg* is activated and *Ubx* and *dpp* are repressed by *abd-A* (8, 20–22).

dpp and wg signaling emanating from the visceral mesoderm induces localized expression of another homeotic gene, labial (*lab*), in the adhering endoderm (20–22). This induction is dependent on *lab* function (12), suggesting that *lab* autoregulation depends on one or both of these signals. We therefore asked whether these signals may have a similar role in *Ubx* autoregulation in the visceral mesoderm.

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MATERIALS AND METHODS

Fly Strains. The same *wg* and *dpp* mutant alleles were used as described (20). Homozygous *wg* mutants were recognized by their altered morphology. For *dpp*, a strain was made that contains the dpp^{s4} allele and a RP transposon (27) on the same chromosome, balanced with a CyO chromosome bearing a β-galactoside (βgal) transposon (for unambiguous identification of homozygous mutants).

Heat-Shock Procedures and Antibody Stainings. To produce wg or dpp protein ectopically, a *hs–wg* strain (28) or a *hs–dpp* strain (29) was used, which in each case was also made homozygous for the RP transposon. Embryos from these strains were heat-shocked two or three times for 20 min at 36°C, 1–3 h before fixation (recovery periods at 25°C). Repetition of the heat shock did not result in qualitative changes of expression patterns but seemed to increase the proportion of embryos showing the effect of ectopically produced *wg* gene product. All effects described below were strictly due to the *hs–wg* transposon and not to the heat shock itself. For antibody staining, a monoclonal antibody against Ubx protein (30) or an antiserum against βgal (Cappell Laboratories), dpp (22), or wg (31) protein was used. We found that the levels of ubiquitous dpp or wg protein in the heat-shocked *hs–wg* or *hs–dpp* embryos, respectively, are below the limit of detection. Stainings were done as described (26). Midguts were dissected for Ubx staining (20) for better inspection.

RESULTS

A short 1.4-kb fragment upstream of the *Ubx* transcription start site (called RP) was sufficient to mediate a *Ubx*-like expression pattern in parasegment (ps) 7 of the visceral mesoderm if linked to a minimal hsp70 promoter and a βgal gene (27) (Fig. 2*a*). βgal expression in ps7 is strictly dependent on Ubx function (27), and the RP fragment must, therefore, contain target sequences for autoregulation.

Unexpectedly, we found that RP-mediated βgal staining was eliminated in *wg* mutant embryos (Fig. 2*c*). Thus, RP-mediated expression depends on an activating function of *wg*. Since the level of wg protein in ps8 of the visceral mesoderm is drastically reduced in *Ubx* mutants (20), this implies that the dependence of the RP pattern on *Ubx* function (27) is at least partly indirect and reflects its dependence on *wg* function.

RP-mediated βgal staining was also virtually undetectable in embryos lacking *dpp* expression in the visceral mesoderm (dpp^{s4} mutants; Fig. 2*b*). Residual levels of staining could be discerned in occasional embryos, presumably reflecting residual levels of *wg* expression in these mutants (20). Since lack of *dpp* function in the visceral mesoderm leads to drastic reduction of wg protein expression in ps8 (20), we cannot be certain from this result whether *dpp* is required in addition to

Abbreviations: βgal, β-galactosidase; ps, parasegment.
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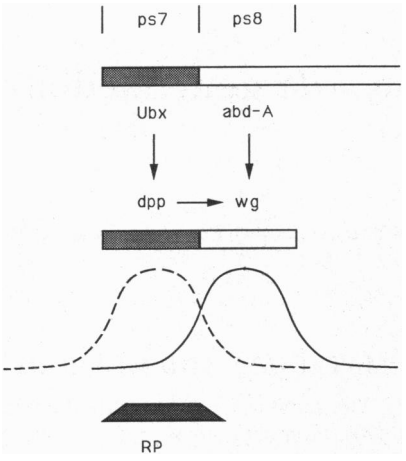

FIG. 1. Expression of *wg* and *dpp* and RP-mediated βgal staining in the midgut visceral mesoderm. Expression of *dpp* in ps7 (shaded bar), dependent on *Ubx* function (expressed in this parasegment; shaded bar), and expression of *wg* in ps8 (open bar), dependent on *abd-A* (expressed in ps8 and posterior regions; open bar) and *dpp* function (8, 20–22). A normal pattern of RP-mediated βgal staining in ps7, trailing into ps8 (shaded area labeled RP; limits of expression blurred), probably as a consequence of activation by simultaneous *wg* and *dpp* signaling, is shown at the bottom. To explain the patterns of RP-mediated βgal staining under various conditions, wg protein is assumed to spread across one parasegment to either side, as indicated. dpp protein may also spread away from ps7 since *wg* expression in ps8 depends on *dpp* function (range of spreading is, therefore, assumed to be similar in both cases). Activation of the RP fragment in ps8 may be low due to competing *abd-A* repression in this parasegment. Note also that *Ubx* expression requires *Ubx* function and that *Ubx* and *dpp* expression is repressed by *abd-A* function (8, 21) (interactions not indicated).

wg for activity of the RP fragment. That this may be the case is indicated by a second weak domain of RP-mediated βgal expression anteriorly in the visceral mesoderm (27), localized in the region in which the gastric caeca form and coinciding, as far as we can tell, with the anterior expression domain of *dpp* in this germ layer (Fig. 3; the latter is probably localized in ps3 as it is anteriorly adjacent to the expression domain of the homeotic gene Sex combs reduced; refs. 21, 22, and 26). Near this, in the foregut visceral mesoderm, is a strong anterior domain of *wg* expression that is independent of *dpp* function (20). Anterior RP-mediated βgal staining in ps3 is not affected by *Ubx* mutation (27); however, it was eliminated in *wg* and in *dpp* mutants (data not shown).

A rule emerges that RP-mediated βgal staining seems to be observed in those visceral mesoderm cells that express *dpp* and that are near cells expressing *wg*. dpp and wg protein spread to neighboring cells (22, 31), the latter apparently across several cells (32), and genetic results implied that wg function spreads anteriorly from ps8, whereas dpp function spreads posteriorly from ps7, in both cases by about one parasegment (20). Since all RP-mediated βgal staining was dependent on wg, and perhaps also on dpp function, we surmised that the RP fragment may be activated in those cells that produce (and/or receive) the dpp protein and that, at the same time, receive high enough levels of the wg protein. We therefore assume that wg protein spreads throughout ps7 (and, for symmetry reasons, throughout ps9; Fig. 1). Furthermore, trailing of βgal staining into ps8 may indicate similar spreading of dpp protein (since *wg* expression depends on dpp function, dpp protein is presumed to spread throughout ps8 and, for symmetry reasons, throughout ps6; Fig. 1). According to this hypothesis, we might expect the wg protein to be limiting anteriorly within ps7 and also posteriorly within ps3 and thus to determine the anterior or posterior limits, respectively, of RP-mediated expression in these positions. Therefore, if we were to provide wg protein ubiquitously, by using a fly strain bearing a *wg* cDNA linked to a heat-inducible promoter (*hs–wg*) (28), we might expect to see these limits shifting away from the normal sources of wg production. Conversely, if we were to provide dpp protein ubiquitously in the same way, we might expect to see these limits shift toward or into the domains of wg production.

We found that βgal staining in ps7 and in ps3 was stronger and that the width of staining in both cases increased when *hs–wg* embryos are briefly heat-shocked two or three times during the last 3 h before fixation (Fig. 4 *a* and *d* compared

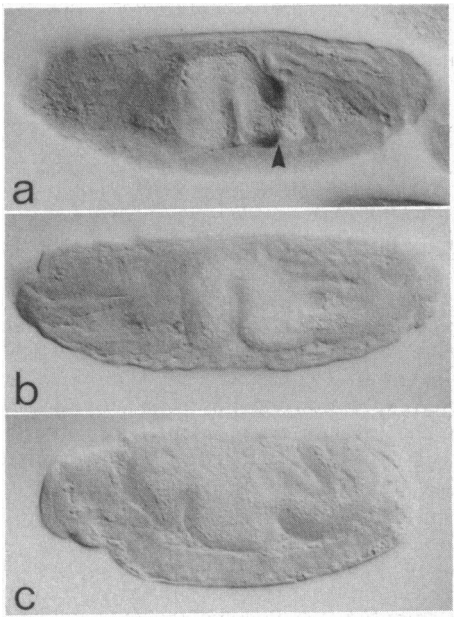

FIG. 2. Dependence of RP-mediated βgal staining on *wg* and *dpp*. Side views of ≈15-h embryos, stained for βgal activity. A strong band of βgal staining in ps7 of the visceral mesoderm (posterior limit of staining at second midgut constriction, indicated by large arrowhead) visible in the wild type (*a*) but virtually undetectable in dpp^- mutants (*b*) and eliminated in wg^- mutants (*c*); note the lack of second midgut constrictions in the mutants. Weak βgal staining in the ps3 region (hardly visible in focal plane of *a*, but see Figs. 3*b* and 4*b*) is completely undetectable in these mutants. Anterior is to the left; dorsal is to the top.

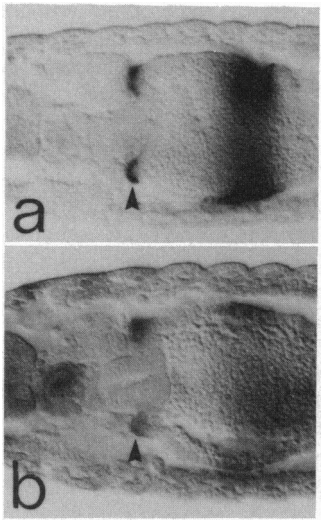

FIG. 3. RP-mediated βgal staining and *dpp* expression in the anterior midgut. Ventral views of anterior midgut region of ≈15-h embryos, stained with βgal (*a*) or dpp antibody (*b*). Staining in both cases is seen in the visceral mesoderm of the budding gastric caeca (arrowheads) and appears to coincide. Anterior is to the left.

FIG. 4. Activating effects of ubiquitous wg and dpp proteins. Side views of RP transformant embryos, ≈13 h (*a–c*) or 14–15 h (*d–f*), also bearing a *hs–wg* (*a*, *d*) or a *hs–dpp* (*c*, *f*) transposon, heat-shocked (*a*, *c*, *d*, and *f*) and stained with βgal antibody. βgal staining in ps7 is expanded anteriorly in the presence of ubiquitous wg protein (open triangles in *a* and *d*) but is expanded posteriorly in the presence of ubiquitous dpp protein (open triangles in *c* and *f*), reaching the first (*a* and *d*) or the third (*c* and *f*) midgut constriction (second midgut constrictions are indicated by arrowheads). Note that βgal staining in the anterior midgut is slightly expanded, probably toward posterior regions, in response to ubiquitous wg protein (compare *a* with *b*). Anterior is to the left; dorsal is to the top.

to *b* and *e*). This increase in width amounted to about one parasegment in the middle midgut, and the expansion of ps7 staining was indeed in the anterior direction, as was seen in embryos of advanced stages where βgal staining extended from the second into the first midgut constriction (Fig. 4*d*), i.e., from ps7 to the anterior limit of ps6 (26). The posterior limit of ps7 staining was unaltered by this treatment. The expansion of βgal staining in the anterior midgut (in ps3) was less obvious but was probably directed posteriorly. These results demonstrate that ectopic wg protein can lead to ectopic activation of RP-mediated expression.

We did a similar experiment, by using instead of the *hs–wg* strain, a fly strain bearing a heat-inducible *dpp* cDNA (*hs–dpp*; ref. 29). We observed slightly increased βgal staining in the middle midgut, which in this case was conspicuously expanded posteriorly from ps7 (Fig. 4 *c* and *f* compared to *b* and *e*). The expansion amounts to about two parasegments as βgal staining reached as far as the third midgut constriction (forming at the limit between ps9 and ps10; ref. 26). In other words, the expansion covered the domain of *wg* expression and an additional parasegment posterior to it (ps8 and ps9). Ectopic βgal staining in ps8 and ps9 was less strong and solid than that in ps6 (Fig. 4 *a* and *d*); however, this was probably due to competing repression mediated by *abd-A* in ps8 and more posterior regions (RP-mediated expression is sensitive to *abd-A* repression; ref. 29). βgal staining in ps3 did not seem to be affected much by ectopic dpp protein, although it may have expanded slightly toward the anterior. Clearly, ectopic *dpp* expression could lead to ectopic RP-mediated expression, confirming our hypothesis that *dpp* acts separately from and in addition to *wg* through the RP fragment to activate its expression.

These results lend support to our hypothesis (Fig. 1) since βgal expression, as predicted, expanded away from the normal sources of wg production in the presence of ectopic wg protein but toward or across these sources in the presence of ectopic dpp protein. This implies that wg and dpp act as signal molecules in the visceral mesoderm to confer and to position RP-mediated expression. The results are also consistent with the hypothesis that wg and dpp protein may spread away from their sources of production (see *Discussion*).

Finally, we asked whether wg signaling and dpp signaling have an activating effect on *Ubx* expression in the visceral mesoderm. *Ubx* expression in this germ layer is indeed somewhat reduced in *dpp* mutant embryos (22) and probably also in *wg* mutants (20) (this reduction, visible in figure 4d of ref. 20, escaped attention as it might have been a secondary side effect of *wg* mutation; however, the results described below suggest that it may in fact be significant). We therefore wondered whether ubiquitous wg or dpp protein would lead to ectopic *Ubx* expression. We stained *hs–wg* and *hs–dpp* embryos with Ubx antibody after heat-shock treatment.

The only effect of ubiquitous dpp protein appeared to be a somewhat enhanced level of *Ubx* expression in the visceral mesoderm (data not shown). We did not see any spreading of Ubx staining toward ps8 and ps9; however, this is perhaps not unexpected since *abd-A* repression determines the posterior limit of *Ubx* expression in this germ layer (8). Repression mediated by *abd-A*, though obviously somewhat "leaky" for RP-mediated expression (29), may well be completely dominant for the whole *Ubx* gene. In contrast, we observed a very clear effect of ubiquitous wg protein on *Ubx* expression: Ubx staining in the visceral mesoderm was increased and widened (Fig. 5*b*, compare to *a*). Its anterior limit was shifted anteriorly by about one parasegment, whereas the posterior limit was unaffected (Fig. 5 *c* and *d*). *Ubx* expression, therefore, behaved like RP-mediated expression under these conditions. As expected from this result, expression of the anteriorly adjacent homeotic gene Antennapedia (26) was repressed in ps6, and the first midgut constriction was missing in some of the heat-shocked embryos. We conclude that the wg signal can result in activation of *Ubx* expression in the visceral mesoderm.

DISCUSSION

We have shown that dpp signaling and wg signaling activate reporter gene expression through upstream control sequences derived from the *Ubx* gene and that wg signaling can lead to ectopic *Ubx* activation in the visceral mesoderm. These results and the fact that *Ubx* expression is reduced in *dpp* and *wg* mutants (20, 22) imply that at least *wg*, and perhaps both *wg* and *dpp*, are upstream controlling genes of *Ubx* in this germ layer. At the same time, these genes are also target genes of *Ubx* in the visceral mesoderm (20–22). In other words, they are part of an indirect autoregulatory loop whose function apparently is to reinforce and maintain *Ubx* expression.

Ubiquitous expression of wg protein leads to a shift of the anterior limit of *Ubx* expression in the visceral mesoderm, providing evidence that wg signaling normally provides positional information for the maintenance of this expression limit (this limit is initially determined by segmentation gene

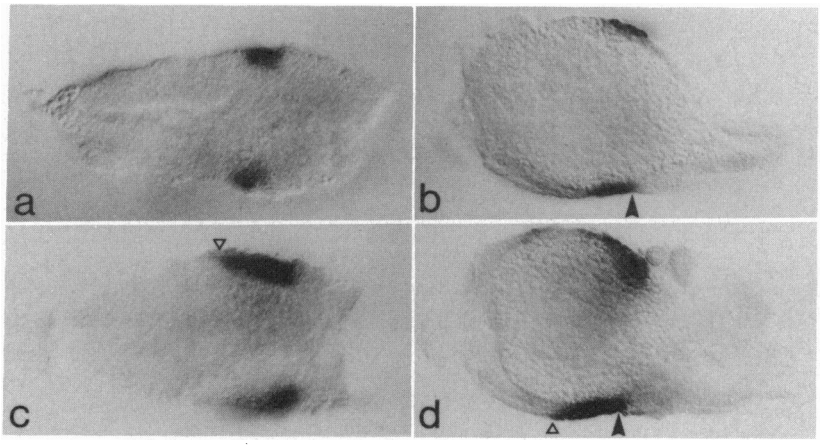

FIG. 5. Ectopic *Ubx* expression in response to ubiquitous wg protein. Dissected midguts from ≈11-h embryos (*a* and *c*) (ventral views) or from ≈13-h embryos (*b* and *d*) (side views), stained with Ubx antibody. Embryos in *c* and *d* bear a *hs–wg* transposon and were heat-shocked before staining. In the presence of ectopic wg protein, anterior limits of *Ubx* expression are shifted anteriorly by about one parasegment (marked by open triangles). Posterior limits of *Ubx* expression (arrowheads) remain unaltered under these conditions and coincide with incipient second midgut constrictions (*c* and *d*). Anterior is to the left (*a–d*); dorsal is to the top (*b* and *d*).

products acting through different *Ubx* control sequences in the early embryo; ref. 33). This result implies that wg protein normally spreads, directly or indirectly, across approximately one parasegment from its source of production, a hypothesis supported by RP-mediated expression in ps9 under conditions of ubiquitous *dpp* expression (the normal domains of *wg* expression in the visceral mesoderm are unaltered under these conditions; ref. 29). It is unclear at present whether dpp protein spreads similarly from its cells of origin: we find (29) that, under conditions of ubiquitous wg protein, *dpp* expression expands anteriorly from ps7, most likely as a consequence of *Ubx* expression (21), which is similarly expanded under these conditions (Fig. 5), and that *dpp* expression in ps3 expands slightly toward posterior regions. In other words, the expansion of RP-mediated βgal expression under these conditions apparently follows the expansion of normal *dpp* expression. Recall also that the RP pattern normally seems to coincide with *dpp* expression, suggesting perhaps that dpp protein does not spread as far as wg protein from its source of production. However, the fact that βgal staining is detectable further away from the source of wg than from the source of dpp production may not be due to a difference in spreading of the two proteins but may simply reflect a stronger responsiveness of the RP fragment to wg- rather than to dpp-mediated activation.

We assume that the activating effects of wg and dpp signaling are mediated by transcription factors (wg and dpp response factors) binding to the RP fragment within the *Ubx* gene. These response factors may be distributed throughout the visceral mesoderm, and their transcriptional activity may be restricted to those cells that receive a minimal level of the corresponding signals. The present data are consistent with the assumption that efficient activation through the RP fragment requires both signals simultaneously. For example, it is possible that one activated response factor acts as a cofactor of the other. However, the residual levels of RP-mediated βgal staining in *dpp* mutants indicate that at least the activated wg response factor may be able to confer on its own a low level of expression. This and additional recent evidence (29) point to the more likely possibility that the two activated response factors act independently but synergistically through RP.

Although dpp and wg signaling are clearly the main activators of RP-mediated expression and although wg signaling has a similar activating effect on *Ubx* expression in the visceral mesoderm, the possibility remains that additional factors binding to sequences outside the RP fragment also play an activating role in the maintenance of *Ubx* expression in this germ layer. One of these may be the Ubx protein itself, which binds to sequences downstream of the *Ubx* transcription start site (34). These sequences are required for βgal expression in ps7 of the visceral mesoderm and appear to function coordinately with the upstream RP sequences (27). If these *Ubx* footprint sequences mediated direct activation by Ubx protein, this would provide an explanation why *Ubx* expression remains strong in *abd-A* mutants (8) although these lack *wg* expression (20), and why *Ubx* expression, though reduced in *dpp* and in *wg* mutants (20, 22), is not completely abolished in *wg dpp* double mutants (data not shown). Maintenance of *Ubx* expression in these mutants suggests redundancy of pathways: *Ubx*-mediated activation may suffice to some extent for *Ubx* maintenance, even if *wg*- and/or *dpp*-mediated activation is lacking. As pointed out above, there may be synergism between the different pathways.

Why are extracellular signals involved in the autoregulation of a homeotic gene? Direct autoregulatory loops can be intrinsically unstable as they are sensitive to fortuitous drops in product concentration (35). Redundancy of factors participating in the autoregulatory loop (e.g., Ubx protein and factors induced by *dpp* and *wg*) may guarantee the reliability of the process. Perhaps more importantly, determinative events often occur in groups of cells (36–38) and, therefore, expression of selector genes (such as *Ubx*) conferring determination must be retained coordinately in groups of cells, implying the need for cell–cell communication (39). Such coordination may be achieved by extracellular signaling mediating autoregulation, as described here for *Ubx*, where a homeotic gene induces signaling to adjacent cells and where its activity in turn is dependent on signaling from adjacent cells.

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