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Dissection of an indirect autoregulatory response of a homeotic *Drosophila* gene

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Homeotic genes often use autoregulation as a mechanism to maintain their expression. Autoregulation of *Ultrabithorax* (*Ubx*) in the visceral mesoderm is at least partly indirect and mediated by extracellular signalling from *wingless* (*wg*) and *decapentaplegic* (*dpp*). *Ubx* controls the localized expression of these two extracellular proteins. Here, we identify separate *wg* and *dpp* response elements within upstream sequences of *Ubx*. Our evidence suggests that there are two distinct response factors each of which, after signal-induced activation, mediates transcriptional activation through its cognate element, whereas each element is recognized by a repressor in the absence of the corresponding signal. We show that the response factors and other components for transmission of the *wg* and, probably, of the *dpp* signal are present throughout the midgut mesoderm. Thus, there may be ubiquitous repression, preventing *Ubx* autoregulation throughout the visceral mesoderm, which is relieved locally by *wg* and *dpp* signalling. Evidently, the two signals convey positional information, allowing visceral mesoderm cells to reassess their position at advanced stages of embryogenesis and to decide whether or not to maintain expression of a homeotic gene.

Key words: decapentaplegic/extracellular signals/indirect autoregulation/*Ultrabithorax*/*wingless*

Introduction

Developmental pathways are determined by the products of selector genes (García-Bellido, 1975) many of which are homeobox genes (Lewis, 1978; Kaufman *et al.*, 1980; reviewed by Lawrence, 1992). These genes are required till late stages of development (Lawrence and Morata, 1976; Morata and García-Bellido, 1976), and positive autoregulation may be used as a mechanism to maintain their activity (García-Bellido and Capdevila, 1978). Positive autoregulation plays a role in the regulation of many selector genes of *Drosophila* (Bienz and Tremml, 1988; Kuziora and McGinnis, 1988; Chouinard and Kaufman, 1991; Heemskerk *et al.*, 1991; Tremml and Bienz, 1992) and may also operate in the control of their mammalian counterparts (Pöpperl and Featherstone, 1992). There is evidence that positive autoregulation of homeobox genes can be direct, i.e. mediated by their products acting through their own control regions (McCormick *et al.*, 1990; Jiang *et al.*, 1991; Regulski *et al.*, 1991; Pöpperl and Featherstone, 1992; Schier and Gehring, 1992).

Based on their analysis of mutant *Ultrabithorax* (*Ubx*) expression patterns in *Drosophila* imaginal discs, Botas *et al.* (1988) suggested that autoregulation of a homeotic gene may require cell-cell communication. Indeed, we found that autoregulation of *Ubx* in the visceral mesoderm (Bienz and Tremml, 1988) is at least partly indirect and mediated by extracellular signalling (Thüringer and Bienz, 1993; see Figure 1). One of the signals involved is encoded by *wingless* (*wg*), a gene whose mammalian counterpart is *Wnt-1* (Cabrera *et al.*, 1987; Rijsewijk *et al.*, 1987). So far, there are four cases where autoregulation of a *Drosophila* selector gene depends on extracellular signalling (Heemskerk *et al.*, 1991; González-Reyes *et al.*, 1992; Tremml and Bienz, 1992; Thüringer and Bienz, 1993). It is intriguing that, in each case, there is a requirement for *wg* function, suggesting that *wg* signalling may play a universal role in the maintenance of selector gene expression.

A second extracellular signal required for *Ubx* expression in the visceral mesoderm (Panganiban *et al.*, 1990) is *decapentaplegic* (*dpp*), a gene related to mammalian TGF-β (Padgett *et al.*, 1987). *wg* and *dpp* are both target genes of *Ubx* in the visceral mesoderm, *dpp* maybe a direct one (Immerglück *et al.*, 1990; Panganiban *et al.*, 1990; Reuter *et al.*, 1990), *wg* an indirect one (Immerglück *et al.*, 1990). Our results suggested that *wg* and *dpp* act through separate pathways, though in a synergistic manner, to mediate indirect *Ubx* autoregulation (Thüringer and Bienz, 1993). Here, we attempt a functional dissection of the *Ubx* upstream fragment which confers *Ubx* autoregulation (Müller *et al.*, 1989). We identify a *wg* and a *dpp* response element within this fragment and we provide evidence that the two elements function independently, although we observe both positive and negative interactions between them.

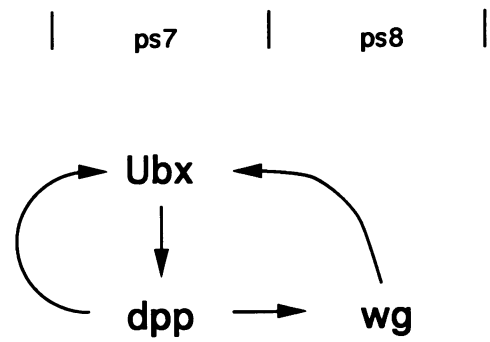

Fig. 1. Indirect autoregulation of *Ubx* in the visceral mesoderm. Schematically depicted are *Ubx* and *dpp* expression in ps7, and *wg* expression in ps8. Positive regulatory interactions between the three genes are indicated by arrows. Note that the indirect autoregulatory loop mediated by *wg* is necessarily based on cell-cell communication (cf. Thüringer and Bienz, 1993; for further explanations and references, see text).

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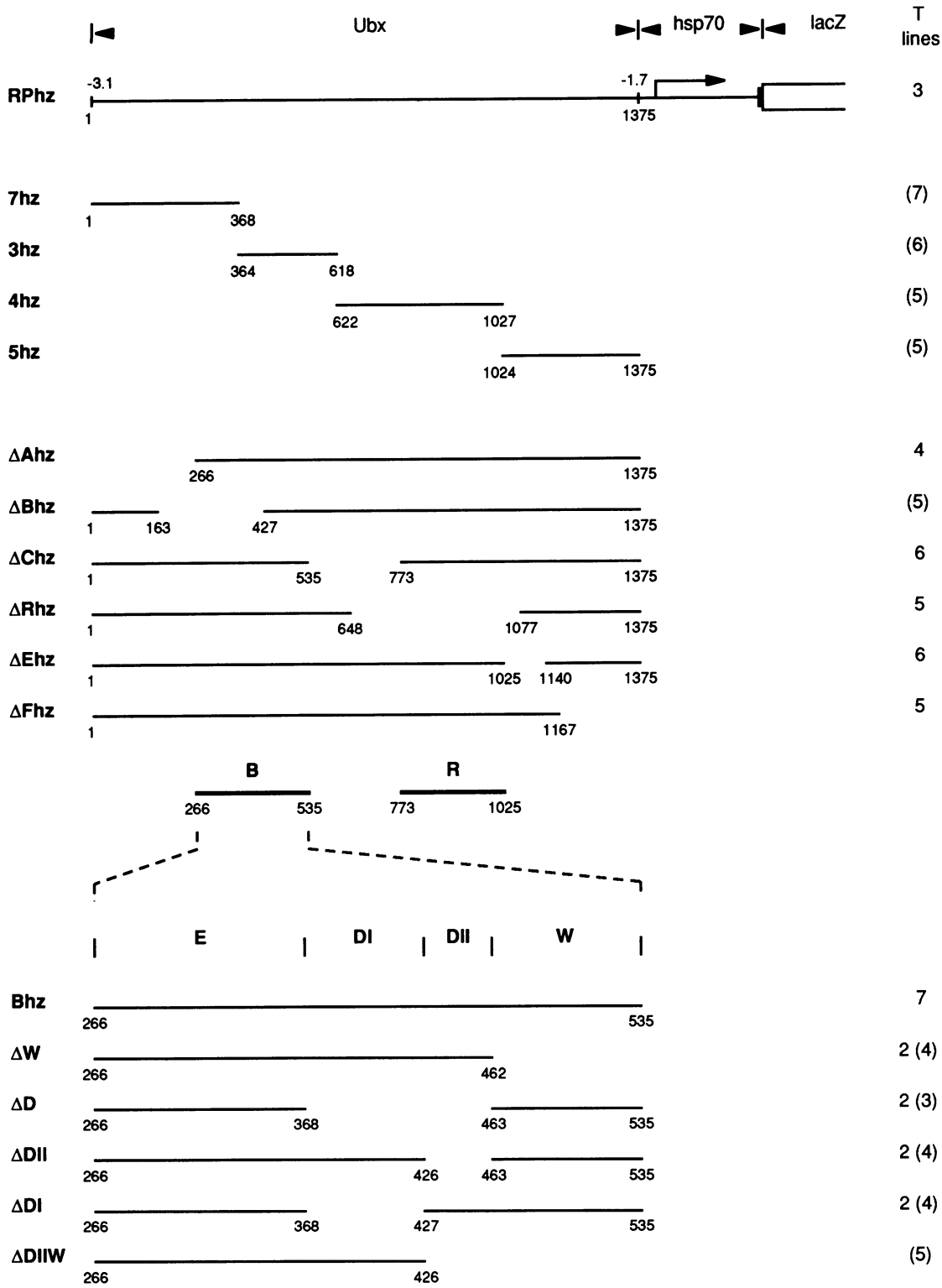

Fig. 2. Maps of RPhz and deletion plasmids. On top, map of RPhz (see also Müller *et al.*, 1989) in HZ50PL (Hiromi and Gehring, 1987); above line, positions of the RP fragment with respect to the *Ubx* transcription start site (*Eco*RI site at position 1, *Pst*I site at position 1370; see Saari and Bienz, 1987). Underneath, maps of 10 deletion mutants of RPhz, names at the left (positions of endpoints given underneath lines). These mutants define two subfragments within RP (B and R) that are required in an activating (B) or repressing fashion (R) for the RP expression pattern (see text). Bottom, maps of six deletion mutants of Bhz, names at the left (positions of endpoints given underneath lines). At the right, number of transformant lines (T) analysed for each construct; numbers without brackets indicate lines with β-gal expression patterns, numbers within brackets lines without any β-gal staining in the visceral mesoderm. For extents of expression patterns mediated by Bhz and its deletion derivatives, see Figure 8a.

Results

A fragment derived from −1.7 to −3.1 kb upstream of the *Ubx* transcription start site (called RP), if linked to an hsp70 promoter and a β-galactosidase (β-gal) gene, confers strong β-gal staining in parasegment (ps) 7 in the visceral mesoderm of transformed embryos (Müller *et al.*, 1989). This β-gal staining mimics *Ubx* expression which is found exclusively

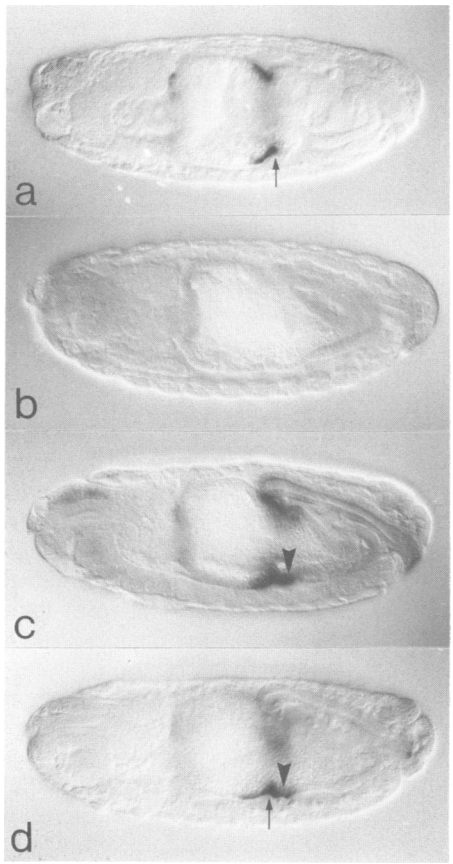

Fig. 3. An enhancer and a repressor element in the RP fragment. Side views of ~13 h old embryos, stained with β-gal antibody, transformed with RPhz (**a**), ΔBhz (**b**), ΔRhz (**c**) or Bhz (**d**); incipient second midgut constrictions in (a) and (d) indicated by small arrows. Note the β-gal staining posteriorly of this constriction (in ps8 and 9; arrowheads) in (c) and (d). Heads to the left, dorsal up (same orientation of embryos in all subsequent figures).

in ps7 in this germ layer (Bienz *et al.*, 1988), and is strictly dependent on *Ubx* function (Müller *et al.*, 1989). We noticed that RP-mediated expression is detectable at low levels in cells outside ps7 in the visceral mesoderm, i.e. in cells that do not express any *Ubx* protein: β-gal staining slightly trails into ps8 and is also seen in ps3; the former, but not the latter is dependent on *Ubx* function (Müller *et al.*, 1989). RP-mediated expression almost precisely coincides with *dpp* expression whose main expression domains in the visceral mesoderm overlap ps3 and ps7 (St Johnston and Gelbart, 1987; Panganiban *et al.*, 1990). Each of these domains is adjacent to or in close proximity to a domain of *wg* expression: *wg* protein is detectable in ps8 as well as in the foregut mesoderm, probably in the region of the anterior limit of ps2 (van den Heuvel, 1989; there is a third domain of strong *wg* expression at the midgut/hindgut junction, however, there is no source of strong *dpp* expression near this third domain). Indeed, most if not all RP-mediated β-gal staining is eliminated in *dpp* as well as in *wg* mutants (Thüringer and Bienz, 1993). *dpp* expression in ps7 and *wg* expression in ps8 of the visceral mesoderm are both dependent on *Ubx* function (Immerglück *et al.*, 1990), and it therefore seemed possible that the RP pattern reflects activation mediated by *dpp* and *wg*, rather than directly by *Ubx* (Thüringer and Bienz, 1993). In order to define these putative *dpp* and *wg* response elements, we started to dissect the RP fragment.

An activating and a repressing element within RP

In a first step, we cut down the 1.4 kb RP fragment into four similarly sized subfragments (Figure 2) and we linked each of these to the hsp70 promoter. Though several independent transformants were isolated in each case, none of them showed any β-gal expression. We proceeded to introduce six different gaps into the RP fragment (Figure 2) in order to identify the regions within this fragment that are required for the RP pattern. Of these internal deletion constructs, four confer a β-gal staining pattern which is indistinguishable from the RP pattern in all respects (Figure 3a). One internal deletion construct, called ΔBhz, does not direct any β-gal expression whatsoever (Figure 3b). Finally, one internal deletion construct, ΔRhz, confers an altered RP pattern. In this case, β-gal staining is equally strong in ps7 and in ps8, and even trails into ps9 at late embryonic stages (Figure 3c); ΔRhz transformants also show β-gal staining in ps3. Thus, these internal deletions define two regions, called B and R, which are necessary for the RP pattern: B contains activating sequences required for all RP-mediated expression, whereas R contains repressor sequences which function to suppress RP-mediated expression in ps8 and 9.

We next asked whether the region indicated by ΔB might be sufficient to mediate β-gal expression in the visceral mesoderm. We linked a fragment overlapping the ΔB deletion, called B fragment (between residues 266 and 535, counting from the *Eco*RI site of the RP fragment; cf. Saari and Bienz, 1987), to the hsp70 promoter and stained transformants of this construct (Bhz) with β-gal antibody. Indeed, these transformants show a β-gal pattern which, as far as we can see, is indistinguishable from that seen in ΔRhz transformants: β-gal staining is most prominent in ps7 and in ps8, but also trails into ps9 and, very weakly, into ps6 (the latter may have been missed in ΔRhz transformants as β-gal staining in these is weaker); there is also weak staining in ps3 (Figure 3d). Clearly, the B fragment is sufficient to mediate an RP-like pattern in the visceral mesoderm.

Separable dpp and wg response elements within the B fragment

Our initial dissection of the RP fragment suggested that the B fragment may consist of multiple activating elements; in particular, the 5′ portion of the latter (called E element, to the left of position 368; Figure 2) appeared to be essential for expression (Figure 2). We made two deletion constructs which either lack the most 3′ portion (ΔW) or a middle portion of the B fragment (ΔD); both of these constructs retain the E element (Figure 2). Both deletion constructs are capable of directing β-gal expression in transformed embryos, although β-gal staining in both cases is weaker compared with B-mediated staining. Also, we isolated transformant lines which did not show any staining at all (Figure 2). Clearly, these B deletions work with somewhat impaired efficiency; however, since we isolated two positive transformant lines showing the same pattern in each case, we think it very likely that the two patterns truly reflect the particular set of *cis*-regulatory elements remaining within the ΔW and ΔD constructs.

β-gal staining in ΔW transformants is mostly confined to ps7 (Figure 4b), although traces of β-gal staining can sometimes be seen in ps6 and 8. As in the case of Bhz transformants, there is also some staining in ps3. In other words, expression mediated by this construct roughly

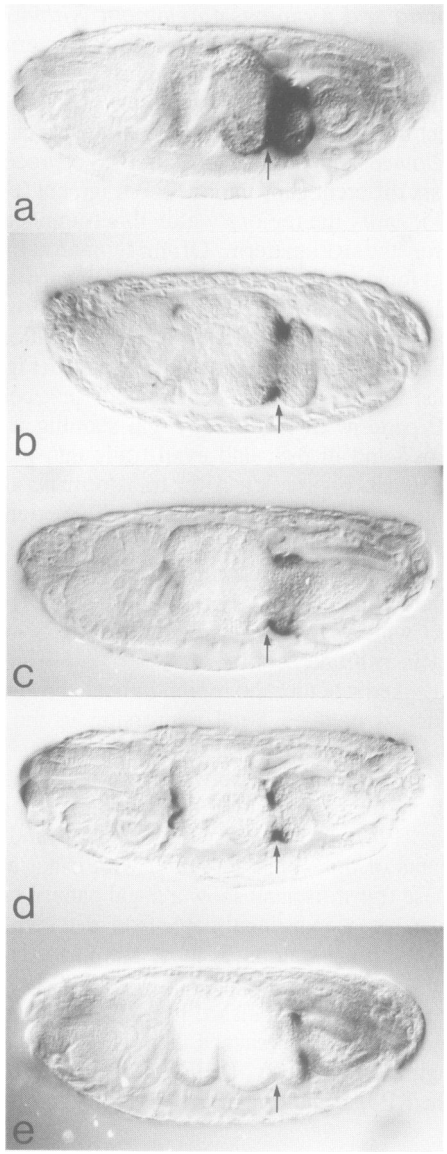

Fig. 4. Dissection of the B fragment. Side views of ~14–15 h old embryos, stained with β-gal antibody, transformed with Bhz (**a**), ΔW (**b**), ΔD (**c**), ΔDII (**d**), or ΔDI (**e**). Midgut constrictions were used to determine approximate expression domains (first constriction coincides with the ps5/6 junction, second constriction, marked by small arrows, with the ps7/8 junction, and third constriction with the ps9/10 junction; midgut mesoderm extends through ps3–12 as well as through some of ps2 and ps13; Tremml and Bienz, 1989). β-gal staining spreads across the second constriction in (a) (ps6–9) and in (d) (strongest staining in ps7 and 8), is mostly confined to the left of this constriction in (b) (to ps7 where *dpp* is expressed) and to the right of this constriction in (c) and (e) (to ps8 where *wg* is expressed, and/or to ps9). There is also some anterior β-gal staining in ps3 (where *dpp* is expressed) in (a) (not visible in view shown; but see Figure 6e), (b) and (d), and in ~ps2 (where *wg* is expressed) in (c) (hardly visible in view shown; but see Figure 6h).

coincides with *dpp* expression in the visceral mesoderm (St Johnston and Gelbart, 1987; Panganiban *et al.*, 1990; see Figure 8a). This suggests that the ΔW construct may be activated in the visceral mesoderm entirely in response to *dpp* signalling.

In contrast, β-gal staining in ΔD transformants is strongest in ps8 and trails somewhat into ps9 and 7 (Figure 4c). In this case, there is weak staining in the foregut mesoderm, probably in ps2, trailing slightly into the midgut mesoderm. β-gal expression mediated by the ΔD construct roughly coincides with *wg* expression in the visceral mesoderm (van den Heuvel *et al.*, 1989; see Figure 8a), suggesting that this construct is activated in the visceral mesoderm entirely in response to *wg* signalling.

The ΔW and the ΔD patterns, if superimposed, roughly add up to the Bhz pattern (apart from the ps2 staining which appears to be suppressed in Bhz transformants), although the latter is much stronger and is also observed more reliably in individual transformant lines than either of the two former. This suggests that the Bhz pattern results from separate, but synergistic activation by each of the two signals. Consistent with this, Bhz-mediated expression, like RP-mediated expression, is dependent on *dpp* as well as on *wg* function: in *dpp* and in *wg* mutants, β-gal staining is much reduced (not shown). Since the ΔW and the ΔD patterns hardly overlap, this indicates that the *wg* and the *dpp* response elements are separable, the former residing within the W sequence, the latter within the D sequence (although it is possible that the *dpp* response element spans the lefthand breakpoint of D at residue 368; see Figures 2 and 8a). The complementarity of the two patterns also indicates that the E element does not contain any further *dpp* or *wg* response sequences, but that this element may contain a general enhancer or a target sequence for a visceral mesoderm-specific factor (Figure 8a). Recall that neither the *wg* nor the *dpp* response element on its own is sufficient to direct any β-gal expression (Figure 2) and, therefore, we assume that the E element somehow cooperates with the two signal response elements.

The D sequence contains an ATTA motif, the core recognition sequence of homeodomain proteins (Kissinger *et al.*, 1989; Ekker *et al.*, 1991). This motif is weakly protected by purified *Ubx* protein (*Ubx* footprint sequence between residues 427 and 457, Figure 2; there are additional and stronger footprint sequences within RP; however, this is the only one in the B subfragment; D.von Kessler and P.Beachy, personal communication). We constructed two further deletion constructs one of which (ΔDII) lacks this *Ubx* footprint sequence as well as five additional 3′ flanking residues; the other deletion construct, ΔDI, lacks the complementary part of the D sequence (Figure 2). Again, we obtained transformants which did not show any β-gal staining, but we also isolated two in each case which showed a consistent, albeit somewhat weak β-gal staining pattern in the midgut mesoderm.

β-gal staining in ΔDII transformants is strongest around the second midgut constriction, posteriorly within ps7 and anteriorly within ps8, but it also trails into ps6 and ps9 (Figure 4d). In addition, there is moderately prominent β-gal staining in ps3. This ΔDII pattern closely resembles, and may even be the same as the Bhz pattern (Figure 4a; see also Figure 8a); the chief difference between the two appears to be the staining intensity, enhanced in the anterior, but reduced in the middle midgut mesoderm in ΔDII compared with Bhz transformants. The ΔDII pattern implies that the *dpp* response element resides within DI rather than in DII.

In ΔDI transformants, we see strongest β-gal staining in ps9 as well as perhaps a hint of staining in ps6 (Figure 4e). There is no β-gal staining in ps7 or in ps3 (the two *dpp* expression domains), consistent with the suggestion that the

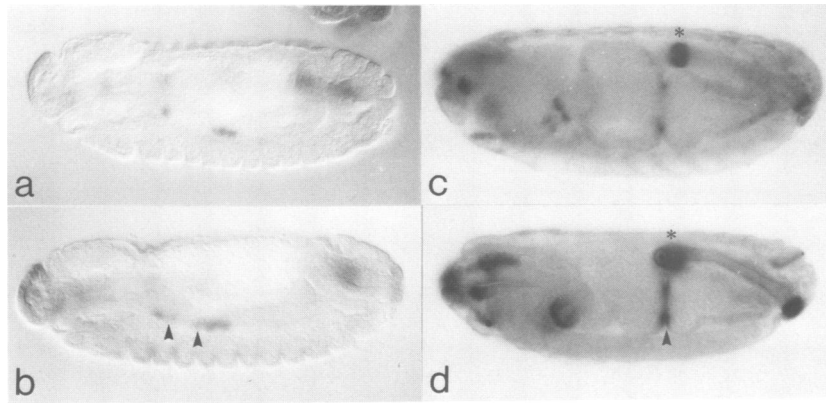

Fig. 5. Effects of ectopic *wg* and *dpp* protein on *wg* and *dpp* expression. (**a** and **b**) ~10 h old embryos bearing a hs-wg transposon, stained with *dpp* antibody after heat-shock treatment. Anterior expansion of *dpp* expression in ps7 by ~one parasegment and slight posterior expansion of ps3 expression domain marked by arrowheads (b). (**c** and **d**) ~15 h old embryos bearing a hs-dpp transposon, stained with *wg* antibody after heat-shock treatment and visualized by brightfield optics. *wg* expression is affected very little under these conditions, but may be slightly stronger and may persist longer (*wg* expression in the hindgut, marked by asterisk, appears to be similarly strong in both cases). Second midgut constriction in (d) marked by arrowhead.

DI sequence is essential for the *dpp* response. The drastic reduction of β-gal staining in ps8 in these transformants indicates that the *wg* response of the ΔDI construct is suppressed at high levels of *wg* signalling; staining in ps8 is evidently restored in ΔD transformants which lack DI as well as DII (see Figure 8a). It therefore seems that addition of the DII sequence to the *wg* response element modifies the activity of this element.

Finally, we tested a construct which contains entirely the DI sequence joined to the E element (ΔDIIW). None of the transformants of this construct showed any detectable β-gal expression (not shown). This points to a functional importance of the DII sequence which, after addition to the ΔDIIW construct (in the ΔW construct), restores expression in ps7 and ps3.

It appears that the DII sequence has different effects, depending on whether it is added to the DI or to the W sequence. These effects are somewhat hard to interpret, and it should be borne in mind that some of the effects of internal deletion mutants could be due to novel juxtaposition rather than lack of sequences. Nevertheless, the results indicate that the DII sequence is not functionally inert. As this sequence consists largely of a homeoprotein footprint sequence, this may explain why DII has variable modifying effects on other response elements, a positive effect if added to ΔDIIW (in ps7) or to ΔDII (in ps7−9) and a negative effect if added to ΔD (in ps8): the effect of the DII sequence may depend on which homeoprotein is bound to it (see Figure 8a).

Repression mediated by the dpp response element

We asked whether the Bhz construct and its derivative deletions could respond to ectopically expressed *wg* and *dpp* protein. For this, we used fly strains containing a *wg* cDNA (Nordermeer *et al.*, 1992) or a *dpp* cDNA linked to a heat-inducible promoter (hs-wg and hs-dpp; see Materials and methods). We previously found that *Ubx* expression in the visceral mesoderm is expanded by one parasegment towards anterior as a result of ubiquitous *wg* expression (Thüringer and Bienz, 1993). Accordingly (cf. Immerglück *et al.*, 1990; Panganiban *et al.*, 1990; Reuter *et al.*, 1990), we find that, under the same conditions, *dpp* expression is expanded by approximately the same extent (Figure 5a and b; for conditions of heat-shock treatments see Materials and methods). Conversely, ubiquitously expressed *dpp* hardly affects *wg* or *Ubx* expression in the visceral mesoderm, though *wg* expression in ps8 may be slightly enhanced and may persist somewhat longer during embryonic development (Figure 5c and d); *Ubx* expression may also be slightly enhanced (not shown). Note that the ubiquitously induced levels of *wg* and *dpp* protein are very low as we cannot detect these proteins outside their normal domains of expression after heat shock.

The *wg* and the *dpp* proteins are capable of spreading to adjacent cells (van den Heuvel, 1989; Panganiban *et al.*, 1990), maybe across several cells (González *et al.*, 1991). Furthermore, *dpp* and *wg* function spread as far as one parasegment away from the source of *dpp* and *wg* production (Immerglück *et al.*, 1990). Therefore, the levels of *wg* or *dpp* protein in parasegments adjacent to their normal sources of production may be as high or higher than the ubiquitous levels of these proteins produced by the heat-shock treatment. In the following experiments, based on heat-induced *wg* or *dpp* protein, we shall therefore pay most attention to β-gal staining appearing in those parasegments which are most distant from the normal *wg* and *dpp* expression domains, i.e. those anteriorly to the first midgut constriction (in ps4 and 5) as well as those posteriorly to the third midgut constriction (in ps10−12).

In Bhz and ΔDII transformants, there is additional β-gal expression after heat-shock induction of *dpp*: in these transformants, we see β-gal staining in ps5, slightly trailing into ps4, as well as in ps10 and 11 (Figure 6a and b; arrowheads). Furthermore, anterior β-gal staining expands into the foregut mesoderm, into ps2, where *wg* is normally expressed. ΔDI transformants respond less to ectopic *dpp*, but they do show slight β-gal staining in the region of ps5 and in ps10 (Figure 6c). ΔW transformants also show additional β-gal staining after heat-shock induction of *dpp*, although in this case, β-gal staining remains confined to ps6−9 and, anteriorly, to ps3 (not shown). In contrast, the ΔD construct does not respond at all to heat-induced *dpp*

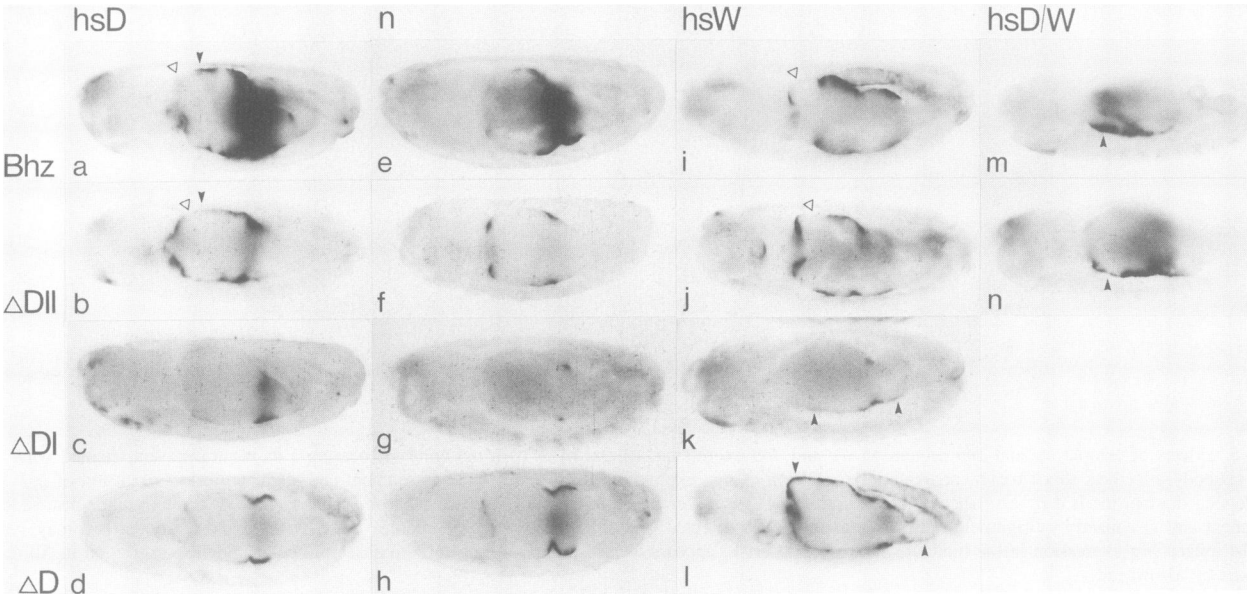

Fig. 6. Response of Bhz and deletion constructs to ectopically expressed *dpp* and *wg* protein. Side views of ~12–14 h old embryos transformed with Bhz (top row), ΔDII (second row), ΔDI (third row) and ΔD (bottom row), after heat shock treatment (except second column, n, no heat shock; first column, hs-dpp; third column, hs-wg; fourth column, hs-dpp + hs-wg). Embryos are viewed with brightfield optics to visualize weak β-gal staining. Ectopic β-gal expression in the regions of ps5 (in **a**, **b** and **k**–**n**) and of ps10 (in k and l) indicated by arrowheads; lack of β-gal staining in the region of ps4/5 indicated by open triangle (a, b, i and j). Note the sharp limits of β-gal staining at the first midgut constrictions in (i) and (j) (to the right of open triangles) and the even β-gal staining throughout the midgut mesoderm in (l).

protein: ΔD-mediated β-gal staining remains unaltered after the heat-shock treatment (Figure 6d).

These results confirm to a large extent our suggestion that the D sequence contains a *dpp* response element and that the latter resides mostly in DI. The DII sequence also seems to confer a weak response to ectopic *dpp*; however, since DII contains a homeoprotein binding sequence, it is possible that this apparent *dpp* responsiveness of DII reflects activation by a *dpp*-activated homeoprotein. We note that the ΔW construct containing a *dpp* response element responds less well to ectopic *dpp* than the Bhz and ΔDII constructs which contain a *dpp* as well as a *wg* response element.

Next, we tested all five constructs under conditions of ubiquitously induced *wg* protein. The most striking response is seen with the ΔD construct: in this case, β-gal staining extends evenly throughout the midgut esoderm (Figure 6l; arrowheads). The ΔDI construct also showed ectopic β-gal staining, intermittently visible throughout ps4–11 (Figure 6k; arrowheads). The other three constructs show less of a response to ubiquitous *wg* protein: in each case, β-gal staining is increased, especially in ps6 and ps9, but remains confined to ps6–9 and to ps3 (Figure 6i and j; staining may trail slightly from ps3 towards posterior in the case of ΔDII and Bhz). We observe sharp limits, coinciding with the first and/or third midgut constrictions, of the β-gal expression domains in the middle midgut of Bhz, ΔDII and ΔW transformants (Figure 6i and j; open triangles indicate the region of ps4/5 where no β-gal staining can be seen). Finally, all constructs except ΔW and ΔDI show β-gal staining throughout the hindgut mesoderm (visible in Figure 6i and l).

These results confirm that the W region contains a *wg* response element which, in the absence of the adjacent *dpp* response element, is capable of responding to *wg* protein evenly throughout the midgut mesoderm. Evidently, in the presence of a linked *dpp* response element, the function of the *wg* response element is suppressed in certain parasegments (in ps4 and 5, and posteriorly to ps9). These correspond to the parasegments that are the most distant from the normal sources of *dpp* production. This points to a repressor acting through the *dpp* response element which, in the absence of *dpp* signalling, prevents the *wg* response element from functioning. Consistent with this, the only construct mediating β-gal expression under normal conditions in ps2, the anterior *wg* expression domain, is ΔD in which the *wg* response element cannot be prevented from functioning by a repressed *dpp* response element as the latter is deleted in ΔD (*dpp* expression in ps3 is less prominent than that in ps7, and the *dpp* signal in this region may therefore not spread very far from its source). This putative repressor is evidently not present in the hindgut mesoderm where most constructs retaining the *wg* response element respond to ectopic *wg* protein.

We wondered whether the *dpp*-mediated repression in ps4 and 5 and in the posterior midgut mesoderm could be relieved by simultaneous ubiquitous production of *dpp* protein. We subjected embryos bearing both the hs-wg and the hs-dpp transposons to the same heat-shock treatment as above. The only transformants that showed a significantly different response under these conditions, compared with their response to ubiquitous *dpp* or *wg* protein only, are the Bhz and the ΔDII transformants: in these, β-gal staining is strong in the anterior midgut mesoderm and appears to be continuous throughout ps3–10, trailing into ps2 and ps11 (Figure 6m and n, compare with a and b, and i and j). Even under these conditions, there is still no β-gal staining in the posterior midgut mesoderm.

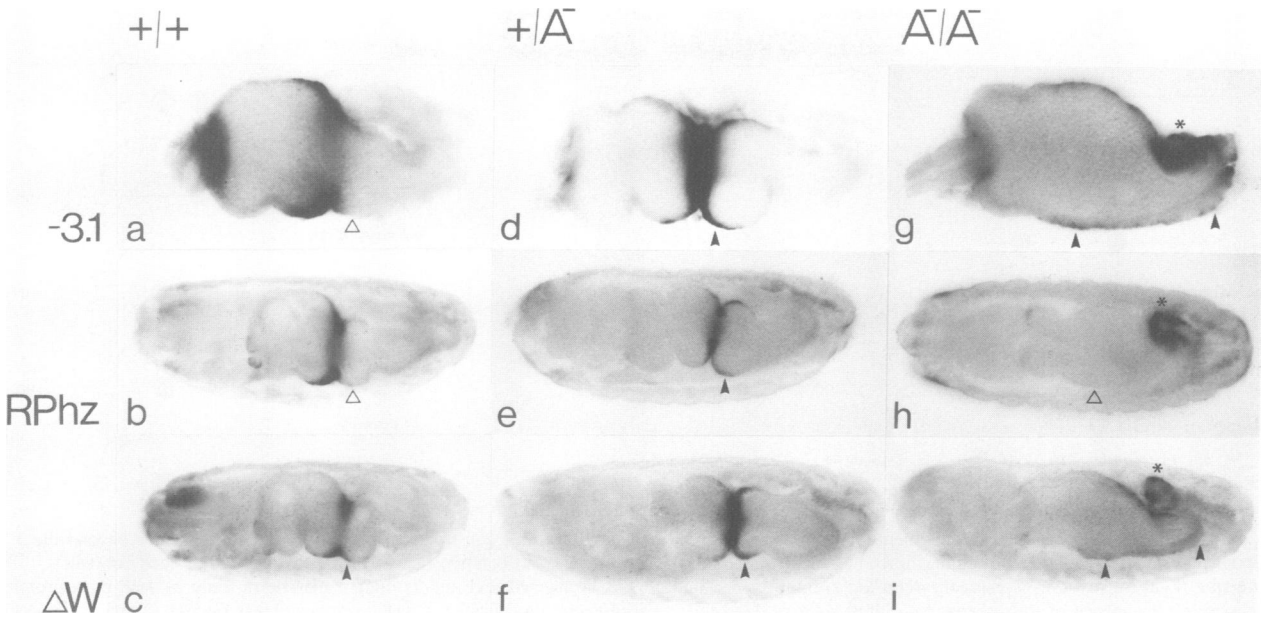

Fig. 7. β-gal expression patterns in *abd-A* mutants. Lateral and dorsolateral views of ~15 h old embryos, stained with β-gal antibody, bearing −3.1 (top row), RPhz (middle row) or ΔW (bottom row). First column, wild-type; second column, putative *abd-A* heterozygotes; third column, *abd-A* homozygotes [lacking second and third midgut constrictions; note also ectopic β-gal expression in most posterior midgut sections, underneath asterisk in (**g**−**i**) (see text and cf. Bienz and Tremml, 1988)]. Embryos are viewed by brightfield optics. β-gal staining is derepressed (indicated by arrowheads in **d** and **e**) posteriorly of the second midgut constriction in *abd-A* heterozygotes, but virtually absent from the same region in the wild-type (open triangles in **a** and **b**). Staining in this region is also seen in heterozygous (**f**, arrowhead) and, to a slightly lesser extent, in wild-type ΔW transformants (**c**, arrowhead). Note that β-gal patterns in *abd-A* heterozygotes are very similar to the patterns in wild-type ΔRhz and Bhz transformants (cf. Figure 3c and d).

β-gal patterns in abdominal-A mutants: evidence for repression mediated by the wg response element

abdominal-A (*abd-A*) is the homeotic gene expressed posteriorly adjacent to *Ubx* in the visceral mesoderm (Tremml and Bienz, 1989). It acts to suppress *Ubx* and *dpp*, thereby determining the posterior expression boundaries of these genes (Bienz and Tremml, 1988; Reuter *et al.*, 1990). *abd-A* repression apparently acts through sequences within a *Ubx* promoter fragment: β-gal staining from a construct containing this fragment (called −3.1; Bienz *et al.*, 1988) is mostly restricted to ps7 in the visceral mesoderm (Figure 7a; weak staining is also seen in ps3), but is derepressed throughout the *abd-A* expression domain in *abd-A* mutants (Bienz and Tremml, 1988; Figure 7g), following *Ubx* and *dpp* derepression. Interestingly, there is a partial derepression of β-gal staining in ps8 and 9 in at least half of the embryos from the *abd-A* cross (Figure 7d). We presume that these are the *abd-A* heterozygotes, indicating that *abd-A* repression of the −3.1 construct is on the brink of functioning in the wild-type and is lost in ps8 and 9 under conditions where the concentration of *abd-A* protein is reduced to half (in the *abd-A* heterozygotes; *Ubx* expression is not derepressed in these heterozygotes; Bienz and Tremml, 1988). It is likely that the loss of *abd-A* repression in ps8 and 9 reflects an activator present in these two parasegments but absent more posteriorly, perhaps the factor activated by *wg* signalling, which competes with the *abd-A*-dependent repressor. The β-gal pattern in *abd-A* heterozygotes is virtually identical to the pattern mediated by constructs lacking the R fragment (in ΔRhz and in Bhz; Figure 3c and d), strongly suggesting that the R fragment is required for *abd-A* repression.

In the case of RP transformants, we observe the same partial derepression of β-gal staining in ps8 and 9 in *abd-A* heterozygotes (Figure 7e, compare with b). This strongly suggests that the RP construct, like the −3.1 construct, responds to *abd-A* repression. Somewhat unexpectedly, we find that RP-mediated expression is undetectable in the middle midgut mesoderm of *abd-A* mutants (Figure 7h; staining in ps3 is unchanged in these mutants, but there is novel β-gal staining in the posterior midgut mesoderm, appearing under conditions where either *Ubx* or *Abdominal-B* or both are expressed in these cells; cf. Bienz and Tremml, 1988; Thüringer, 1992). As *wg* function is essential for RP-mediated expression (Thüringer and Bienz, 1993), the loss of β-gal staining in the middle midgut mesoderm probably reflects the loss of *wg* expression in this region in *abd-A* mutants (Immerglück *et al.*, 1990).

If *abd-A* repression depends on the R fragment, none of the small deletion constructs ought to be sensitive to *abd-A* repression. Indeed, all of these but one (ΔW) mediate expression in ps8 and/or 9 and therefore show 'partial derepression' in the wild-type. ΔW transformants show very little expression in ps8 and none in ps9 in the wild-type (Figure 7c). However, this probably reflects the lack of a *wg* response element in this construct. As expected, there is very little difference between the β-gal patterns in ps8 and 9 in the wild-type compared with *abd-A* heterozygotes (Figure 7c and f; the very slight enhancement of β-gal staining in ps8 and 9 in *abd-A* heterozygotes may indicate a slight responsiveness of ΔW to *abd-A* repression). Most strikingly, in *abd-A* homozygotes, the normal levels of ΔW-mediated β-gal staining can be observed from ps7 through the posterior midgut mesoderm, i.e. throughout the *abd-A*

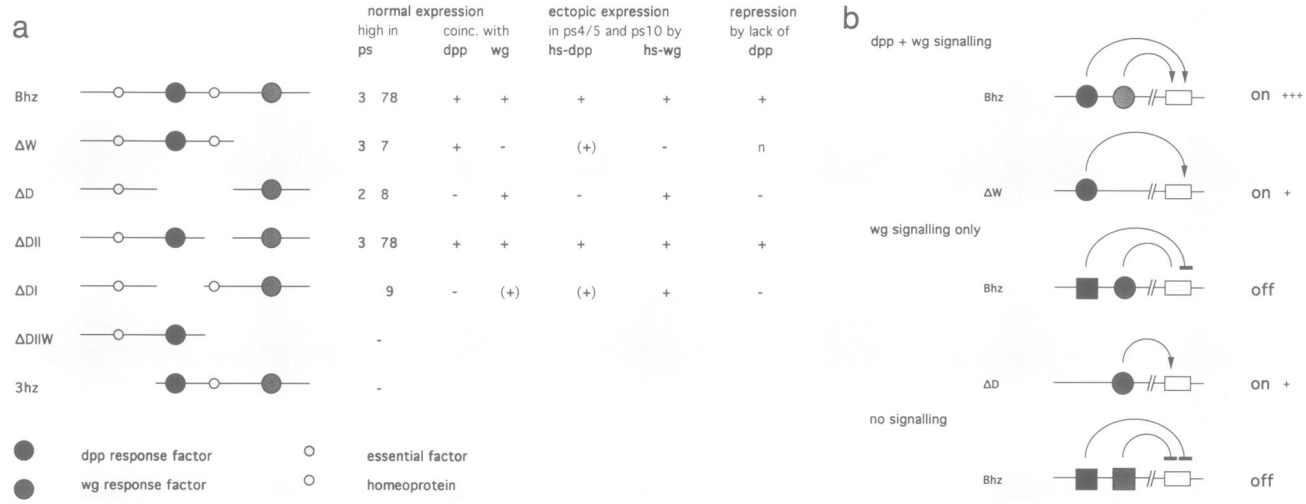

Fig. 8. Regulatory elements in the B fragment and their interactions. (**a**) maps of various constructs (names at the left; 3hz extends beyond the 3′ endpoint indicated; cf. Figure 2) defining functional elements within the B fragment and putative factors binding to these. For each construct conferring β-gal expression, given at the right are normal expression domains in anterior and middle midgut mesoderm (only ps with most prominent expression), coincidence of this expression with *dpp* (in ps3 and ps7) or *wg* expression (in ps2 and ps8), responsiveness (in ps4, 5 and 10) to ectopic *dpp* and *wg* expression, and repression, as inferred from the results, in the absence of *dpp* signalling; brackets indicate partial coincidence or partial responsiveness (see also text). Repression by lack of *dpp* is only seen in constructs responsive to both *dpp* and *wg* (Bhz and ΔDII; the partial responsiveness to *dpp* of ΔDI may reflect responsiveness to a *dpp*-activated homeoprotein, see text) and correlates with presence of the DI sequence (the *dpp* response element); this repression cannot be determined in the case of ΔW (n) as this construct is hardly responsive to ectopic *dpp* protein outside ps6–9. (**b**) Schematic representations of transcriptional activity (on; +++, strong, + weak) or inactivity (off) of β-gal constructs as a consequence of signalling by both *wg* and *dpp* (top), by *wg* only (middle) or in the absence of either signal (bottom). Activated response factors (black and stippled circles, as in **a**) and their activating effect (arrows), corresponding inactive response factors (black and stippled squares) and their repressing effect (bars) on the linked promoter (open square). Each signal is presumed to activate one response factor which in its inactive form (in the absence of signalling) may act as a repressor (conversely, it is possible that activator and repressor in each case are distinct molecules, recognizing nearby or overlapping DNA sequences, and that each signal induces DNA binding of either the activator or the repressor; see text). Note the synergistic interaction between the two activated factors (strong transcriptional activation in the presence of both factors, e.g. in Bhz, top; weak transcriptional activation by just one factor, e.g. in ΔW, top, or in ΔD, not shown). Negative interaction is observed in the absence of one of the signals (e.g. in ps4/5 and posteriorly to ps10 in heat-induced hs-wg embryos), only if both response elements are present (in Bhz, but not in ΔD, middle): an inactive response factor binding to its cognate element acting as a repressor, thereby preventing the other bound response factor, although activated, from mediating transcriptional activation. A similar scenario may be found in the presence of *dpp*, but absence of *wg* signalling (e.g. in *abd-A* mutants, not shown; see text).

expression domain (Figure 7i). This β-gal pattern is similar to that in −3.1 transformants (and to *Ubx* and *dpp* derepression) in *abd-A* mutants. There are a number of possible explanations for this result. The one we favour is the possibility that a repressor acts through the *wg* response element in the absence of *wg* signalling (see below).

Discussion

A wg and a dpp response element

Our functional analysis of the RP fragment from the *Ubx* gene provides strong evidence that this fragment contains a *wg* and a *dpp* response element. We have argued, based on spatially distinct expression patterns of β-gal constructs and on differential responsiveness of these constructs to ectopic *wg* and *dpp* protein, that the W sequence contains a fully functional *wg* response element, whereas the D sequence contains at least part of a *dpp* response element. The latter appears to reside in DI, the upstream part of the D sequence, though it is possible that this element spreads across the lefthand breakpoint of DI (the D/E breakpoint; Figure 2).

We have shown that each of these elements is capable of functioning in the absence of the other, strongly suggesting independence of the *wg* and the *dpp* signalling pathways. We assume that at least one factor binds to each of these elements, a putative *wg* and a putative *dpp* response factor (Figure 8a). According to our evidence, these factors are present throughout the midgut mesoderm, but they do not confer transcriptional activation in the absence of signalling. Signal-mediated activation of these response factors may induce their binding to DNA or, should they be bound to DNA constitutively, may induce their interaction with other proteins (proteins bound near the transcription start site or 'coactivators'; e.g. Pugh and Tjian, 1990) which, directly or indirectly, causes transcriptional activation. Alternatively, it may be that the response factors are repressors present throughout the midgut mesoderm and bound to DNA. Signal-induced activation of these repressors would cause their dissociation from DNA and would therefore result in the relief of ubiquitous repression. Activation in this case would be due to a general activator(s) binding within or outside the two response elements [e.g. the activator(s) binding to the E element]. There is a third possibility, uniting aspects of both the above ones: the response factors might act as repressors prior to their signal-induced activation by virtue of their constitutive binding to DNA (Figure 8b). In the following paragraphs, we shall provide arguments in favour of this third possibility.

Repression mediated by the two response elements

Although the *wg* and *dpp* response elements function independently, we observe positive as well as negative interaction between the two elements if they are linked.

Evidence for synergistic action between the two elements stems from the observation that the pattern conferred by the linked elements is much stronger (and shows a more reliable penetrance among individual transformant lines) than either pattern conferred by just one of them. Also, expression mediated by the linked elements (in Bhz transformants) is dramatically reduced if only one of the two corresponding signals is available (in *wg* mutants). Evidence for negative interaction was revealed under certain circumstances (see below) where one element prevents the activity mediated by the other. This implies that there are repressors acting through these elements, and that a repressor bound to one element competes at short range (cf. Small *et al.*, 1991) with an activator bound to the other for transcriptional activation of the linked gene (Figure 8b).

Evidence for a repressor acting through the *dpp* response element comes from the most striking of our results that the ubiquitous response of the *wg* element to *wg* signalling (Figure 6l) is prevented in certain regions of the visceral mesoderm by a linked *dpp* response element. Repression is observed in those regions in which there is apparently no *dpp* signalling (in ps4 and 5 and posteriorly to ps9, i.e. those most distant from the normal sources of *dpp* production). Most of this repression can be overcome by ubiquitous production of *dpp* protein (Figure 6m and n). Furthermore, the target sequence of the repressor is probably located in DI rather than in DII, because the repressing effect is lost in ΔDI, but not in ΔDII transformants (compare Figure 6j and k). Therefore, the repressor target sequence is closely linked if not coinciding with the target sequence for the *dpp* response factor. Taken together, this suggests the possibility that this repressor may be identical with the inactive *dpp* response factor: the latter may be constitutively bound to DNA and act as a repressor in the absence of *dpp* signalling (Figure 8b). Note that inactive *dpp* response factor may also prevent β-gal expression in ps2, the anterior *wg* domain, as expression in this parasegment is only seen in the absence of the *dpp* response element (in ΔD transformants; Figure 4c).

If the inactive *dpp* response factor acted as a repressor, a consequence of this might be that *dpp*-mediated activation may be somewhat difficult to achieve as the activated factor presumably competes for DNA binding with inactive repressing factor. In particular, a pulse of ubiquitously expressed *dpp* protein may not be sufficient to convert enough inactive factor into active factor, an insufficiency that might be further aggravated if the inactive *dpp* response factor was abundant. This may provide an explanation why there is no response to a pulse of ubiquitous *dpp* protein in the posterior midgut mesoderm (a region most distant from the normal *dpp* sources; Figure 6m and n) and why the *dpp* element (in the ΔW construct) does not respond ubiquitously to such a pulse.

Is there evidence for a similar repressing effect of inactive *wg* response factor? Unfortunately, due to the limited responsiveness of the *dpp* element to ubiquitous *dpp* protein, we were unable to detect a repressing effect of the *wg* response element in our heat shock experiments. However, the analysis in *abd-A* mutants may have revealed such an effect: in the absence (ΔW construct), but not in the presence of the *wg* response element (RP construct), β-gal expression is seen throughout the middle and posterior midgut mesoderm, following *Ubx* and *dpp* derepression in these mutants (Figure 7i). It is possible that the lack of RP-mediated expression in the middle midgut mesoderm of *abd-A* mutants (Figure 7h) reflects repression due to absence of *wg* signalling in these mutants. If true, we would expect to see expression in this region in these mutants either in the absence of the *wg* response element (ΔW construct) or in constructs (−3.1 construct and *Ubx* gene) with a strong responsiveness to *dpp*-mediated activation, perhaps assisted by additional activators (see below), which may be capable of out-competing *wg*-mediated repression. Support for this view that *wg* signalling may result in the relief of repression (i.e. that the inactive *wg* response factor may be a ubiquitous repressor) comes from recent results by Siegfried *et al.* (1992) who identify a kinase that might mediate the effects of *wg* signalling in the embryonic epidermis.

What might be the consequence of inactive response factors acting as repressors? It has been proposed that *dpp* acts as a morphogen in the early embryo, i.e. that subtly different concentrations of *dpp* protein induce cells to follow different developmental pathways (Ferguson and Anderson, 1992). In amphibian development, it appears that isolated blastoderm cells interpret different levels of activin, a *dpp*-related factor, in that they switch on or off different genes in response to different concentrations of exogenously applied activin (Green and Smith, 1990; Green *et al.*, 1992). Furthermore, Green *et al.* (1992) have demonstrated that the response of individual genes to increasing activin concentrations is very sharp. If the inactive *dpp* and *wg* response factors were repressors, as suggested above, this might provide a sharpness of the response, reflecting a critical change of balance between activated and inactive response factor depending on the intensity of the corresponding signal. Although the patterns of our constructs usually show blurred expression limits, we found that the limits of the Bhz and ΔDII patterns become sharp upon ubiquitous production of *wg* protein (Figure 6i and j), implying a sharp response to *dpp* signalling at least under these conditions. The limits of *Ubx* expression in the visceral mesoderm are sharp (Tremml and Bienz, 1989) and it is conceivable that the sharp anterior limit reflects at least in part a sharpness of the response to *wg* (though not to *dpp*) signalling (Thüringer and Bienz, 1993).

Redundant pathways

We have demonstrated that there are at least two separate activation pathways, mediated by *dpp* and by *wg* signalling, which act through *Ubx* upstream control sequences. There is strong evidence that the same two pathways also act on *Ubx* expression in the embryonic visceral mesoderm. First, efficient expression of *Ubx* in this germ layer is dependent on *dpp* (Panganiban *et al.*, 1990) and probably on *wg* function (Immerglück *et al.*, 1990; the partial reduction of *Ubx* expression in both *dpp* and *wg* mutants is visible, though not pointed out by Immerglück *et al.*, 1990, in their Figure 4, and may indeed be significant in the light of our recent results). Secondly, ubiquitously produced *wg* protein causes ectopic activation of *Ubx* in the visceral mesoderm (Thüringer and Bienz, 1993; see below).

We have asked whether *Ubx* protein might act directly, in addition to *wg* and *dpp*, to provide transcriptional activation. In an attempt to answer this, we analysed the

response of most of our constructs to ubiquitous *Ubx* protein, by using a hs-Ubx strain (González-Reyes *et al.*, 1990). However, the resulting patterns could be explained entirely by the assumption that they were due to ectopic *dpp* protein (induced throughout ~ps2–7 under these conditions; Reuter *et al.*, 1990, and our own observations). In particular, constructs with or without the homeoprotein binding site in the DII sequence were equally responsive to ubiquitous *Ubx* protein, and, therefore, there is no evidence from these experiments to suggest that *Ubx* protein might be acting directly through these sequences.

However, it is possible that *Ubx* protein acts directly by binding to the strong homeoprotein footprint sequences which were found immediately downstream of the *Ubx* transcription start site (Beachy *et al.*, 1988; Biggin and Tjian, 1988). These sequences are required for β-gal expression in the visceral mesoderm of transformed embryos (Müller *et al.*, 1989). If they were the target sites for direct *Ubx* activation, this might provide an explanation why expression of the RP construct is eliminated in *abd-A* mutants, whereas expression of the −3.1 construct and of *Ubx* itself remains strong in these mutants: it is conceivable that *Ubx*-mediated activation can compensate to a large extent for the loss of *wg*-mediated activation in *abd-A* mutants. Furthermore, this putative direct action of *Ubx* protein might assist *dpp*-mediated activation, and the two activation pathways together might counteract and overcome the putative *wg*-mediated repression.

There appears to be redundancy between the *wg* and *dpp* activation pathways, although it seems unlikely that they can fully substitute for one another. If, in addition, there was direct *Ubx*-mediated activation, this amounted to an apparent 3-fold redundancy. Such redundancy may be necessary for reliability of important control mechanisms, e.g. for maintenance of homeotic gene expression (Thüringer and Bienz, 1993). A strong argument for the necessity of redundant pathways in the control of genes such as *Ubx* has been made previously by Laney and Biggin (1992).

Positional signalling and respecification of position

The ubiquitous response of ΔD transformants throughout the visceral mesoderm to ectopic *wg* protein implies that the machinery needed for the production as well as for the reception and transmission of the *wg* signal is present in all cells of the midgut mesoderm. Evidently, the only spatial determinant of ΔD-mediated expression in the visceral mesoderm is the presence of *wg* protein. Perhaps the most important conclusion from this is that *wg* protein itself conveys positional information in this germ layer. The same argument can be made for *dpp* protein which, in a large part of the visceral mesoderm, clearly also conveys positional information. Since the two proteins are secreted and therefore have the potential to spread (cf. González *et al.*, 1991; Panganiban *et al.*, 1990), their concentration might be expected to decrease with increasing distance from their cells of origin. Our β-gal patterns support this notion: they imply that the response to *wg* and *dpp* signalling decreases with increasing distance from the signal sources.

This function of positional signalling mediated by *wg* may also be important with respect to *Ubx* expression in the visceral mesoderm whose anterior expression boundary shifts towards anterior, away from the source of *wg* signalling, if *wg* protein is produced ectopically (Thüringer and Bienz, 1993). On the basis of this, we have argued that the level of *wg* signalling becomes limiting in the cells near this anterior boundary and thus determines whether or not these cells maintain *Ubx* expression. Positional signalling by *wg* is also evident in the pattern of *labial* expression in the adhering endoderm: the stimulating effect of *wg* on *labial* expression is strongest in those cells that are nearest the source of *wg* signalling, and weakest in those cells that are more remote from this source (Immerglück *et al.*, 1990; see also Tremml and Bienz, 1992). Finally, *wg* signalling in the epidermis appears to convey position in a similar way: if *wg* protein is produced ectopically, the posterior limit of *engrailed* expression is shifted towards posterior, i.e. expands away from the normal source of *wg* signalling (Nordermeer *et al.*, 1992). Moreover, stable maintenance of *engrailed* expression in an individual epidermal cell appears to be dependent on proximity of this cell to the source of *wg* signalling (Vincent and O'Farrell, 1992; see also Heemskerk *et al.*, 1991).

We have provided evidence that the *wg* and the *dpp* signals convey positional information in the visceral mesoderm. This implies a function of these signal pathways in respecifying position. Such respecification of position during advanced development may serve as a 'proof-reading' mechanism which leads to a redefinition of particular groups of cells earmarked to maintain expression of a particular selector gene and which also eliminates mistakes (Heemskerk *et al.*, 1991; Vincent and O'Farrell, 1992). It appears that *wg* signalling may have a unique function in stimulating autoregulation of selector genes in cells adjacent to its source of production, a function that has cropped up in several different situations (see Introduction) and that may be used in yet more situations during *Drosophila* development. Finally, determinative events often occur in groups of cells (Gehring, 1967; García-Bellido *et al.*, 1973; Gurdon, 1988; Gurdon *et al.*, 1993), and indirect autoregulation mediated by extracellular signalling may be the mechanism used to guarantee coordinated maintenance of selector gene expression in groups of cells (Thüringer and Bienz, 1993).

Materials and methods

Fly strains

The following mutant alleles were used: *abd-A*^{M1} (Casanova *et al.*, 1987); *dpp*^{shv4} (St Johnston *et al.*, 1990); *wg*^{cx4} (Baker, 1987). The hs-wg strain contains one copy of a heat-inducible *wg* cDNA balanced with a TM3 chromosome (Nordermeer *et al.*, 1992). Two hs-dpp strains were used, one of which is homozygous for the *dpp* transposon on the second, one on the third chromosome. For the hs-dpp construct, a 2.2 kb *Bam*HI–*Ssp*I fragment from a *dpp* cDNA was cloned into the unique *Bgl*II and *Stu*I sites of the polylinker in the transformation vector CaSpeR HS (V.Pirotta, unpublished) containing the hsp70 promoter and the w^+ gene. In the case of hs-Ubx, a strain was used in which the transposon is inserted on the second chromosome and balanced with CyO (González-Reyes *et al.*, 1990).

Crosses and identification of homozygous mutants were done as described (Bienz and Tremml, 1988).

Plasmids

The −3.1 and the RP constructs were previously described (Bienz *et al.*, 1988; Müller *et al.*, 1989). Each of the derivative deletion constructs, like the RP construct, is based on HZ50PL (Hiromi and Gehring, 1987), i.e. the various *Ubx* control fragments are linked to an hsp70 TATA box.

For the 3–7hz series (Figure 2, top), the following subfragments from −3.1 were inserted into a Bluescript vector (bs) in which the *Xho*I site was deleted (cf. Müller *et al.*, 1989): a 370 bp *Xba*I (from polylinker)–filled-in *Spe*I fragment (7hz) into bs cut with *Xba*I and *Apa*I (recessed); a 260 bp *Spe*I–recessed *Bgl*I fragment (3hz) into bs cut with *Spe*I and *Eco*RV; a 400 bp recessed *Bgl*I–*Cla*I fragment (4hz) into bs cut with *Eco*RV and

*Cla*I; a 350 bp filled-in *Cla*I−*Pst*I fragment (5hz) into bs cut with *Sma*I and *Pst*I. These subfragments were then cloned as *Xba*I−*Kpn*I fragments into *Xba*I/*Kpn*I cut HZ50PL.

For the ΔA−ΔFhz series, the 1.4 kb *Xba*I−*Pst*I fragment from −3.1 was subcloned into bs and into bsΔ (cut with *Eco*RV and *Apa*I, recessed and religated), and 3′ (bs) as well as 5′ deletions (bsΔ) were made with exonucleases 3 and 7; deletion endpoints were determined by sequencing. For ΔChz, ΔRhz and ΔFhz, *Dra*III−recessed *Kpn*I fragments from individual 3′ deletion plasmids were cloned into individual 5′ deletion plasmids (in bsΔ) cut with *Sac*I (recessed) and *Dra*III (Figure 2). For ΔBhz and ΔEhz, individual 5′ and 3′ fragments were combined in the same way, after the following subcloning steps into bs (5′ part) or bsΔ (3′ part): a 180 bp *Xba*I−*Bal*I fragment into bs cut with *Xba*I and *Apa*I (recessed) and a 950 bp *Pvu*II−*Pst*I fragment into bsΔ cut with *Bst*XI (recessed) and *Pst*I (ΔBhz); a 1040 bp *Xba*I−filled-in *Cla*I fragment into bs cut with *Xba*I and *Apa*I (recessed) and a 230 bp filled-in *Spe*I−*Pst*I fragment into bsΔ cut with *Bst*XI (recessed) and *Pst*I (ΔEhz). For ΔAhz, a 1.1 kb *Sna*BI−*Pst*I fragment from −3.1 was subcloned into *Sma*I/*Pst*I cut bsΔ (called bsΔA). All six deletion fragments were inserted as *Xba*I−*Kpn*I fragments into HZ50PL.

For Bhz, a 270 bp *Sna*BI−*Kpn*I fragment from the 5′ deletion plasmid (in bsΔ) used for ΔChz was inserted into HZ50PL cut with *Not*I (filled-in) and *Kpn*I. For ΔW, a 200 bp *Xba*I−*Nla*IV fragment from bsΔA was first subcloned into bsΔ cut with *Xba*I and *Eco*RI (filled-in) and then inserted as a *Xba*I−*Kpn*I fragment into HZ50PL. For ΔD, a 100 bp *Sna*BI−*Kpn*I fragment from the bs intermediate used for 7hz was inserted into HZ50PL cut with *Not*I (filled-in) and *Kpn*I (5′ part), and an 80 bp *Nla*IV−*Kpn*I fragment from the ΔChz 5′ deletion plasmid (see Bhz) was inserted into HZ50PL cut with *Not*I (filled-in) and *Kpn*I; an *Xho*I−filled-in *Asp*718 (cutting the *Kpn*I site) fragment from the former (5′ part) was joined with a filled-in *Xba*I−*Xho*I fragment from the latter HZ50PL plasmid (3′ part) to create the final ΔD HZ50PL plasmid (*Xho*I is a unique site within the *ry* gene of HZ50PL). For ΔDII, an 170 bp *Xba*I−*Pvu*II fragment from bsΔA was inserted into HZ50PL cut with *Xba*I and *Sac*II (recessed); an *Xho*I−filled-in *Asp*718 fragment from this HZ50PL plasmid was joined to the same filled-in *Xba*I−*Xho*I fragment as above (3′ part of ΔD) to create the final ΔDII HZ50PL plasmid. For ΔDI, a 110 bp *Pvu*II−*Asp*718 fragment from the ΔChz 5′ deletion plasmid (see Bhz) was inserted into HZ50PL cut with *Not*I (filled-in) and *Asp*718, and a filled-in *Xba*I−*Xho*I fragment from this HZ50PL plasmid was joined as a 3′ part to the same *Xho*I−filled-in *Asp*718 fragment as above (5′ part of ΔD) to create the final ΔDI HZ50PL plasmid. For ΔDIIW, an 170 bp *Xba*I−*Pvu*II fragment from bsΔA was inserted into HZ50PL cut with *Xba*I and *Sac*II (recessed).

Transformations and analysis of β-gal expression

Several individual transformed lines were isolated for each construct, strains homozygous for the transposon were made, and embryos were stained with a polyclonal antibody against β-gal protein (Cappell) as described (Bienz *et al.*, 1988; Tremml and Bienz, 1989). For each construct, at least two independent lines showed the same β-gal staining pattern in the visceral mesoderm; staining elsewhere in the embryo (e.g. in the epidermis or in the somatic mesoderm) was occasionally observed. During microscopy, DIC optics were used if β-gal staining was strong; but we also used bright-field optics routinely to ensure detection of weak β-gal staining. In some cases, midguts were dissected after staining for better inspection (cf. Immerglück *et al.*, 1990).

For assessment of position in the visceral mesoderm, landmarks such as the midgut constrictions and the junctions between the various gut sections (foregut, middle and posterior midgut, hindgut) were mostly used (cf. Tremml and Bienz, 1989). The anterior expression domains of *wg* and *dpp* were estimated on the basis of these landmarks, but were not precisely mapped with respect to parasegmental limits.

Heat shock procedures and analysis of stained embryos

We used a standard heat-shock treatment throughout, consisting of three subsequent 20 min heat shocks at 37°C (fly vials immersed in a waterbath) followed by 40 min recovery periods at room temperature. Embryos were fixed and stained, 1 h after the last recovery period, with antibodies against β-gal (Cappell), *wg* (van den Heuvel *et al.*, 1989) or *dpp* protein (Panganiban *et al.*, 1990) as described (Tremml and Bienz, 1989). We previously found that the number of heat shocks given did not qualitatively change the resulting β-gal expression patterns (Thüringer and Bienz, 1993). Heat-shock control experiments were done with embryos that did not contain any heat-inducible transposon, to ensure that the resulting pattern alterations were not due to the heat-shock treatment itself.

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