Transcriptional repression due to high levels of Wingless signalling

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Extracellular signals can act at different threshold levels to elicit distinct transcriptional and cellular responses. Here, we examine the transcriptional regulation of the Wingless target gene *Ultrabithorax* (*Ubx*) in the embryonic midgut of *Drosophila*. Our previous work showed that *Ubx* transcription is stimulated in this tissue by Dpp and by low levels of Wingless signalling. We now find that high levels of Wingless signalling can repress *Ubx* transcription. The response sequence within the *Ubx* midgut enhancer required for this repression coincides with a motif required for transcriptional stimulation by Dpp, namely a tandem of binding sites for the Dpp-transducing protein, Mad. Indeed, Wingless-mediated repression depends on low levels of Dpp, although apparently not on Mad itself. In contrast, high levels of Dpp signalling antagonize Wingless-mediated repression. This suggests that transcriptional activation of *Ubx* is subject to competition between Dpp-activated Mad and another Smad whose function as a transcriptional repressor depends on high Wg signalling. Finally, we show that Wingless can repress its own expression via an autorepressive feedback loop that results in a change of the Wingless signalling profile during development.

Keywords: endoderm induction/signalling thresholds/Smad/transcriptional repression/Wnt

Introduction

Wingless (Wg) is a Wnt signal with pivotal control functions in all developing germ layers of *Drosophila*. It controls the patterning of the epidermis in the larva (e.g. Nüsslein-Volhard and Wieschaus, 1980; Baker, 1987) and adult (e.g. Sharma and Chopra, 1976; Peifer *et al.*, 1991; Phillips and Whittle, 1993; Struhl and Basler, 1993; Couso *et al.*, 1994; Brook and Cohen, 1996; Jiang and Struhl, 1996; Lecuit and Cohen, 1997), and also the patterning of internal cell layers such as the mesoderm (Baylies *et al.*, 1995) and endoderm (Immerglück *et al.*, 1990; Hoppler and Bienz, 1995). In some of these events, Wg does not simply control binary decisions, but functions at distinct signalling thresholds to determine multiple cell fates (Hoppler and Bienz, 1995; Lawrence *et al.*, 1996;

Zecca *et al.*, 1996; Lecuit and Cohen, 1997; Neumann and Cohen, 1997).

Wg signals to the nucleus through a cascade of transducing molecules which are conserved between species (reviewed by Cadigan and Nusse, 1997). The downstream (nuclear-proximal) part of this pathway is becoming increasingly well understood. In particular, it has been established that all known effects of the Wg signal are mediated by the cytoplasmic factor Armadillo (Arm), which becomes stabilized on signalling and translocates into the nucleus (Noordermeer *et al.*, 1994; Peifer *et al.*, 1994; Siegfried *et al.*, 1994; Orsulic and Peifer, 1996). Nuclear Arm is a transcriptional co-activator of a high-mobility group (HMG) domain protein called dTCF (T cell factor, or Pangolin), the ultimate target factor for Wg signalling (Brunner *et al.*, 1997; Riese *et al.*, 1997; van de Wetering *et al.*, 1997). dTCF is the only known transcriptional response factor for Wg, and the genetic analysis suggested that this factor is an obligatory Wg target mediating all effects of Wg signalling (Brunner *et al.*, 1997; van de Wetering *et al.*, 1997). This raises the question of how multiple cell fate decisions are implemented by multiple thresholds of a single signal which targets a single transcription factor.

We began to address this question by asking whether all effects of Wg signalling are mediated by dTCF in the *Drosophila* midgut. This tissue consists of an outer cell layer, the visceral mesoderm, and an inner layer, the endoderm or midgut epithelium, which is induced by the outer one (Biens, 1997). Wg plays a key part during this induction. First, it synergizes with Decapentaplegic (Dpp), a TGF- β -like secreted signal, within the outer cell layer to stimulate transcription of the homeotic gene *Ultrabithorax* (*Ubx*) (Thüringer *et al.*, 1993). This stimulatory effect of Wg is conferred by dTCF (Riese *et al.*, 1997; van de Wetering *et al.*, 1997). Secondly, together with Dpp, Wg acts on the inner cell layer to stimulate transcription of *labial* (*lab*), a homeotic gene that subsequently determines cell differentiation in the larval gut (Hoppler and Bienz, 1994). Notably, high levels of the same signalling molecule, Wg, repress *lab* transcription in this cell layer (Hoppler and Bienz, 1995). Thus, in the endoderm, two signalling thresholds specify distinct transcriptional activities and cell fates.

Here, we show that Wg can repress transcription in the visceral mesoderm too: at high signalling levels, Wg represses transcription of *Ubx* and also its own expression. Interestingly, Wg-mediated repression of the *Ubx* midgut enhancer also depends on *dpp*, and requires an enhancer motif that is part of the Dpp response sequence within this enhancer. Furthermore, we show that high Dpp signalling antagonizes Wg-mediated repression. This leads to a molecular model of how Wg-mediated repression may be affected by Dpp signalling.

Fig. 1. Transcriptional repression of *Ubx B* by high Wg levels. Side views of embryos bearing *Ubx B* and *hs-wg*, ~12 (left-hand column) or ~14-hour-old (right-hand column), stained with antibody against lacZ; (a and b) no heat shock, (c–j) after heat shocks as indicated on the right. Transcriptional repression due to high Wg is indicated by open triangles (c–e and g), and ectopic transcriptional stimulation by arrowheads (c–j). The middle gut constrictions are marked by vertical bars, and the endogenous Wg sources in the wild type by asterisks; heads to the left, dorsal up.

Results

High Wg levels repress *Ubx* transcription

Previous studies on the *Ubx* midgut enhancer called *Ubx B* revealed that this enhancer is only active in cells of the visceral mesoderm which experience both Wg and Dpp signalling (Thüringer *et al.*, 1993). In the absence of Wg pathway activity, transcription from this enhancer is much reduced (Thüringer *et al.*, 1993; Yu *et al.*, 1996). The stimulatory effect of Wg on *Ubx B* is mediated by the Wg response sequence (WRS) in this enhancer, a TCF binding site; if this site is mutated, the mutant enhancer can no longer be stimulated by Wg (Riese *et al.*, 1997; see Figure 3k). However, we noticed that the mutant (like the wild-type) enhancer still responds to Wg: there is repression of the enhancer in cells overlapping the Wg source [parasegment (ps) 8 of the visceral mesoderm; indicated by an asterisk in Figure 1a and b] after mild overexpression of Wg throughout the midgut (Thüringer *et al.*, 1993; Riese *et al.*, 1997; open triangle in Figure 1g). We presumed this repression to be due to Wg signalling reaching particularly high levels in ps8 cells under these overexpression conditions. Consistent with this, we found that *shaggy/zeste white-3* (*sgg*) mutants (which mimic constitutive activation of the Wg pathway) lacked *Ubx B* expression in the posterior part of the midgut despite showing strong *Ubx B* stimulation more anteriorly (Yu *et al.*, 1996). This suggests that the *Ubx* enhancer is transcriptionally repressed in the posterior midgut by high Wg signalling levels.

To pursue this, we produced various levels of Wg throughout the midgut, using a *hs-wg* transposon and applying different heat-shock regimes (see Materials and methods), and we monitored the effects of these conditions on transcription mediated by the *Ubx B* enhancer. The most extreme conditions produced a β -galactosidase (lacZ) staining pattern similar to that produced by sustained GAL4-mediated Wg expression (using the mesodermal driver line 24B.GAL4, and UAS.Wg): lacZ staining is evenly strong throughout the anterior part of the midgut, but is undetectable posteriorly to the middle gut constriction (Figures 1c–e, 3b, 4b and 5c; the middle constriction, indicated by vertical bars, marks the normal boundary between ps7 and 8; it is not affected by Wg overexpression). In other words, lacZ staining is strongly induced in ps2–6 (arrowheads in Figure 1c), but completely repressed in ps8 and 9 (open triangles in Figure 1c–e, compare with a and b). The stimulation in the anterior midgut is partly due to the overexpressed Wg and partly to endogenous Dpp, which is ectopically activated throughout this region by constitutive Wg signalling (Yu *et al.*, 1996).

Moderate heat-shock regimes produced a mixture of lacZ staining patterns. They range from a trace of staining posterior to the middle gut constriction (Figure 1f) to substantial staining in the posterior midgut with a staining gap in ps8 (Figure 1g, open triangle), and occasionally without such a gap (Figure 1h). In each case, lack of staining within the ps7–9 region signifies repression of transcription due to overexpressed Wg, since *Ubx B* mediates strong expression throughout this region under

Fig. 2. Posterior derepression of *Ubx* RP in *abd-A* and Wg pathway mutants. Side views of ~14-h-old embryos bearing *Ubx* RP, stained with antibody against lacZ; (a) *dpp*^{+/+} (similar to wild type); (b) *abd-A*^{M1/+}; (c) *wg*^{cx4/+}; (d) *dTCF*^{2/+}; (e) *wg*^{cx4/wg} and (f) *dTCF*^{2/dTCF}. Note the increased width of *Ubx* RP expression in the *wg* and *dTCF* homozygous embryos [(e) and (f), compare with (a); arrowheads point to posterior derepression; residual middle gut constriction in *dTCF* embryo indicated by vertical bar]. Symbols and orientations as in Figure 1.

normal conditions (Thüringer *et al.*, 1993). We found that the extent of repression correlates with the strength of the heat-shock regime (see Materials and methods). In particular, four consecutive brief heat shocks spaced by 2 h never caused any repression (Figure 1i and j), although this condition can stimulate *Ubx* B expression (Figure 1j, arrowheads). On the other hand, one longer heat shock 2 h prior to fixation regularly caused repression near the endogenous Wg source (Figure 1g, open triangle). No repression was observed with an early heat shock applied 4 h prior to fixation (not shown, but similar to Figure 1h), but the same early heat shock followed by a second heat shock 2 h later produced significantly more repression than just the latter on its own (Figure 1e, compare with g). This strongly suggests that the extent of repression correlates with the cumulative levels of Wg expression rather than with a particular time window of Wg production. Thus, the Wg expression levels appear to be a critical determinant of whether *Ubx* B transcription is stimulated or repressed.

Involvement of Arm and dTCF in Wg-mediated repression

We have reported that a 1.4 kb enhancer fragment called *Ubx* RP (which includes *Ubx* B) closely resembles *Ubx* expression in the visceral mesoderm as it directs lacZ expression almost exclusively in ps7 (Thüringer and Bienz, 1993; Figure 2a). Transcription of *Ubx* RP, like that of *Ubx* B and of *Ubx* itself, requires both Wg and Dpp signalling, and can be ectopically activated by these signals (*Ubx* RP expression is expanded anteriorly by Wg, and posteriorly by Dpp; Thüringer and Bienz, 1993). We noticed previously that, in embryos that are heterozygous for *abdominal-A* (*abd-A*), *Ubx* RP expression is posteriorly expanded throughout ps8 and 9, mimicking the expression pattern of *Ubx* B (Thüringer *et al.*, 1993). *abd-A* is the homeotic gene expressed posteriorly to *Ubx*; it represses *Ubx* (Bienz and Tremml, 1988), but stimulates *wg* expression in ps8 (Immerglück *et al.*, 1990; Reuter *et al.*, 1990; Figure 6). Evidently, repression of *Ubx* RP posterior to ps7 is very sensitive to the levels of Abd-A, or to a

product such as Wg, whose expression depends on *abd-A*. Notably, deletion of a small internal fragment of *Ubx* RP (distinct from *Ubx* B) also causes derepression of *Ubx* RP in ps8 and 9, indicating that this fragment contains target sequences for Abd-A- and/or Wg-mediated repression. *Ubx* RP is thus responsive to this repression (albeit easily derepressible), while *Ubx* B has lost the ability to be repressed in ps8 and 9 under normal conditions.

We explored the dosage sensitivity of *Ubx* RP to determine whether repression of this faithful *Ubx* enhancer depended on the dosage of Arm and dTCF, i.e. of Wg pathway components that are known to mediate the stimulatory effects of Wg signalling. We examined the *Ubx* RP expression pattern in embryos heterozygous for *wg*, *arm* and *dTCF*. We found that each of these conditions produced a derepression of lacZ staining posterior to the middle gut constriction similar to that seen in *abd-A* heterozygotes (Figure 2b–d, arrowheads). In contrast, *Ubx* RP staining was not derepressed in *dpp* heterozygotes (Figure 2a, open triangle) nor in heterozygotes for *mothers against dpp* (*mad*) (not shown), a gene required for the transduction of the Dpp signal (Sekelsky *et al.*, 1995; Newfeld *et al.*, 1996). These results strongly suggest that the dosage-sensitive derepression of *Ubx* RP in ps8 and 9 observed in *abd-A* heterozygotes is at least partly due to reduced levels of Wg or of its transducing components. This implies that Arm and dTCF are necessary for this repression. Their requirement could be direct or indirect and mediated by a target gene of Arm–dTCF.

We also examined the RP staining pattern in *wg* and *dTCF* homozygous embryos. We found not only loss of staining intensity, reflecting loss of Wg stimulation in these mutants, but also strikingly expanded staining in *wg* (Figure 2e) and *dTCF* homozygotes (Figure 2f). Moreover, the *dTCF* mutants whose overall morphology is comparatively normal and which occasionally even show a residual middle gut constriction (Figure 2f, vertical bar), presumably due to perdurance of maternal dTCF, reveal that there is derepression in the ps8/9 region (Figure 2f, arrowhead). Clearly, the RP enhancer becomes derepressed beyond its normal ps7 expression domain due to loss of Wg pathway

activity in these mutants. This provides confirmatory evidence that high levels of Wg signal transduction near the Wg source repress *Ubx* RP transcription in the wild-type.

WRS-R, a response sequence for Wg-mediated repression

The staining pattern of *Ubx* RP in the *dTCF* mutants implicates dTCF in the Wg-mediated repression. However, this role of dTCF could be indirect and be due, for example, to a stimulatory effect of *dTCF* on endogenous Wg expression (see below). To address the question whether dTCF, in addition to conferring Wg-induced stimulation of transcription, might also confer directly the Wg-induced repression, we asked whether this repression requires TCF binding sites.

Our previous results strongly indicated that this is not the case: the gap of lacZ-staining in response to Wg overexpression is seen with all three mutant enhancers bearing different mutations in the TCF B site (B4, BG and BL; Riese *et al.*, 1997; Figure 3k). Indeed, *Ubx* B4 is as fully repressed in the posterior midgut of 24B.GAL4/UAS.Wg embryos (Figure 3d) as is the wild-type enhancer (Figure 3b; the same is also true for BG and BL; not shown). However, there is another TCF-binding site in the *Ubx* B enhancer, 37 residues upstream of TCF B, namely TCF A (Figure 3k). Indeed, bacterially expressed mouse LEF-1 binds to both sites with comparable affinity (not shown). A mutant enhancer with base substitutions within TCF A (called B3) shows much reduced stimulation of transcription after Wg overexpression (Riese, 1997). Evidently, dTCF binding to this site contributes to the positive response of the enhancer to Wg. B3, like B4, is fully repressed by high Wg levels (not shown).

We thus generated a double-mutant enhancer (called B3B4) devoid of functional TCF binding sites, and investigated whether this enhancer would still be repressible by high Wg. To our surprise, B3B4 was totally inactive. None of our lines showed any lacZ staining whatsoever at any stage of embryogenesis. The mutant construct was clearly present in these lines, as judged by a number of control tests (see Materials and methods). No staining was seen even if B3B4 was exposed to high Dpp or high Wg levels. This demonstrates that these binding sites are not only essential for the positive response to Wg signalling, but are also fundamental for enhancer activity per se [see Bienz (1998) for discussion of TCF functions that are signal-independent]. Unfortunately, this makes it impossible to test whether TCF binding sites are involved in the negative response to high Wg.

We thus asked whether we could identify another enhancer motif required for this repression (to be called WRS-R). We tested each substitution mutant previously made in *Ubx* B (Eresh *et al.*, 1997; Riese, 1997; Szüts *et al.*, 1998) in 24B.GAL4/UAS.Wg embryos to see whether any of these were derepressed posteriorly to the middle gut constriction despite high Wg. In these embryos, *Ubx* B is expanded throughout the anterior midgut where it responds to the ectopic Wg and to endogenous Dpp (*dpp* is ectopically activated throughout this region by high Wg pathway activity; Yu *et al.*, 1996), whereas it is fully repressed posterior to the middle gut constriction (Figure 3b, compare with a). Some of the mutant enhancers examined showed moderately strong lacZ staining in the

posterior midgut of 24B.GAL4/UAS.Wg embryos (BM1, Figure 3f, compare with e; B5 and BC, not shown; see also Materials and methods). In each case, staining posterior to the constriction was considerably weaker than it was anteriorly, indicating that these enhancers still respond partially to Wg-mediated repression. A fourth mutant enhancer showed very strong posterior staining despite high Wg (BM2; Figure 3h, compare with g, b and d). In BM2 embryos, lacZ staining is evenly strong throughout the midgut, indicating that this mutant enhancer can no longer be repressed by high Wg. Two internal deletions of the BM2 region of the enhancer (Δ D and Δ DI; Thüringer *et al.*, 1993) also showed lacZ staining in the posterior midgut (Figure 3j, compare with i and h; Δ DI not shown), whereas the complementary deletion to Δ DI, namely Δ DII (Thüringer *et al.*, 1993), produced lacZ staining exclusively in the anterior midgut (not shown). Taken together, the BM2 mutation identifies a WRS-R in *Ubx* B, and the deletion mutants suggest that the Δ DI region (encompassing BM2 and 44 flanking residues) is the only part of the enhancer which contains a WRS-R (summarized in Figure 3k).

Which sequences are mutated in BM2, and in BM1, BC and B5? *Ubx* B contains a tandem of two binding sites for Mad (MadA and MadB; Kim *et al.*, 1997; Figure 3k). Remarkably, all four mutant enhancers that show loss of repression despite high Wg have at least one Mad-binding site mutated: BC carries a mutated MadA site, BM1 and B5 a mutated MadB site, and BM2 (which has lost all ability to respond to high Wg) has both binding sites for Mad mutated (Szüts *et al.*, 1998). The BM2 mutation also abolishes the positive response to Dpp (Szüts *et al.*, 1998) and is thus part of the Dpp response sequence of *Ubx* B (DRS). Thus, the WRS-R overlaps the DRS in *Ubx* B and appears to consist of two Mad binding sites.

***dpp* is required for Wg-mediated repression**

The sequence of the WRS-R suggests that Mad, or a Mad-related protein, may be involved in the Wg-mediated repression. However, it is known that Mad-like proteins are not nuclear in the absence of signalling; indeed, their translocation to the nucleus depends on phosphorylation by membrane receptors on binding to TGF- β -type signals (Massagué *et al.*, 1997; see also Newfeld *et al.*, 1996). Therefore, it is likely that Mad-like proteins would be available for enhancer binding only in Dpp-stimulated cells. We thus tested whether Wg-mediated repression of *Ubx* B can occur in *dpp* mutants and, as a control, in *wg* mutants (note that *dpp* mutants contain only traces of Wg; Immerglück *et al.*, 1990).

As previously observed (Riese *et al.*, 1997), lacZ-staining from *Ubx* B is very low in *wg* mutants (Figure 4c, compare with a). However, after Wg overexpression throughout the mesoderm (with 24B.GAL4), we found strong stimulation of *Ubx* B expression throughout the anterior midgut (Figure 4d, arrowhead, compare with b), but barely any lacZ-staining posterior to the 'bulge' (Figure 4d, vertical bar) which is observed in *wg* mutants in a location where the middle gut constriction would normally form (see Tremml and Bienz, 1989). In fact, we occasionally observed a residual incipient midgut constriction in this region, and also some restoration of

Fig. 3. A response sequence for Wg-mediated repression in *Ubx* B. (**a–j**) Side views of 12- to 14-h-old embryos bearing *Ubx* B or mutant versions of this enhancer (as indicated on the left), as well as 24B.GAL4 only (left-hand column) or 24B.GAL4/UAS.Wg (right-hand column), stained as in Figure 1. Symbols and orientations as in Figure 1. (**k**) Top, layout of enhancer sequence encompassing WRS and DRS (TCF- and Mad-binding sites underlined; note that the palindromic CRE is also part of the DRS; Szüts *et al.*, 1998); underneath, summary of enhancer mutants and their abilities to respond to Wg and Dpp (see also Eresh *et al.*, 1997; Riese *et al.*, 1997; Szüts *et al.*, 1998). BM2 defines the WRS-R.

Fig. 4. *dpp* is required for Wg-mediated repression. Side views of ~14-h-old wild-type and mutant embryos, as indicated on the left, bearing *Ubx B* and 24B.GAL4 only (**a**, **b**, **e** and **g**), or 24B.GAL4 as well as UAS.Wg (**b**, **d**, **f** and **h**), stained as in Figure 1; **a** and **b**, wild-type; **c** and **d**, *wg^{ex4}/wg^{ex4}*; **e** and **f**, *dpp^{s4}/dpp^{s4}*; **g** and **h**, *Df(2L)JS17/mad^{l2}*. Note that rudimentary middle gut constrictions are observed in **d**, **g** and **h** (approximate positions marked by vertical bars); also, the *Ubx B* reporter line used here is expressed at levels higher than that used in the other experiments and shows some 'leakiness' of repression in the posterior midgut [visible in (**b**) and (**d**)]. Other symbols and orientations as in Figure 1.

segmentation which is completely lost in *wg* mutants, suggesting that the mesodermally expressed Wg rescues some of the *wg* mutant phenotypes (see also Sampedro *et al.*, 1993). Clearly, Wg-mediated repression of *Ubx B* in the posterior midgut can occur in the absence of endogenous Wg.

dpp mutants lack the middle gut constriction and show much reduced lacZ staining from *Ubx B* (Figure 4e, compare with **a**), the strongest staining being in ~ps8 where there is residual endogenous Wg expression (Immerglück *et al.*, 1990). As expected, *dpp* mutants which carry 24B.GAL4 and UAS.Wg do not show any stimulation of lacZ staining in the anterior midgut (Figure 4f); however, lacZ staining is clearly expanded towards the posterior in these mutants (Figure 4f, arrowhead), beyond the region where the middle gut constriction would normally form. There is no observable repression of *Ubx B* in the posterior midgut of these *dpp* mutants which express high levels of Wg. We conclude that *dpp* is required for Wg-mediated repression of *Ubx B*. Evidently, low levels of Dpp suffice for this as there is no detectable Dpp protein in the region of ps8 and 9 (Panganiban *et al.*, 1990) where Wg represses *Ubx B*.

We also asked whether Wg-mediated repression of *Ubx B* depends on *mad*. We found that lacZ staining from *Ubx B* was reduced in *mad* mutant embryos (Figure 4g), as in the *dpp* mutants. However, unlike *dpp* mutants, the *mad* mutant embryos bearing 24B.GAL4 and UAS.Wg showed repression of *Ubx B* in the posterior midgut (Figure 4h, open triangle). They also show moderate stimulation of *Ubx B* in the anterior midgut (Figure 4h, arrowhead), and a residual middle gut constriction (Figure 4g and h, vertical bars), suggesting that they may retain

some *mad* function due to perduring maternal Mad. Nevertheless, our results strongly suggest that *mad* is not required for Wg-mediated repression of *Ubx B*.

High Dpp levels prevent Wg-mediated repression

We note that only some of the cells near the Wg source (those in ps9) show Wg-mediated repression, while others equidistant to this source (those in ps7) do not (see Figures 2 and 3). One distinguishing factor between these two cell groups is their stimulation by Dpp: cells in ps7 constitute the Dpp source and thus experience high levels of Dpp, while those in ps9 are distant from this source and experience, at most, low levels of this signal (see above; Figure 7). We thus surmised that, while low levels of Dpp are necessary for Wg-mediated repression, high levels of Dpp may prevent *Ubx B* from being repressed by high Wg.

We tested this by co-expressing Wg and Dpp in the mesoderm (with 24B.GAL4) and by monitoring *Ubx B* transcription. If Dpp is overexpressed alone throughout the mesoderm, the most noticeable stimulation of lacZ staining is in midgut regions that are near strong Wg sources (Figure 5e, arrowheads, compare with **a**), while Wg overexpression alone produces strong staining where endogenous Dpp is ectopically activated (Figure 5c). However, on co-expression of both signals, we find extensive lacZ staining almost throughout the midgut (Figure 5g, arrowheads), including the posterior midgut, which shows no staining if Wg alone is overexpressed (Figure 5c, open triangles). These results establish that high Dpp levels effectively antagonize Wg-mediated repression.

A similar observation is made if *Ubx* expression is monitored under these conditions. Wg alone stimulates

Fig. 5. Dpp antagonizes Wg-mediated repression of *Ubx*. Side views of ~14-h-old embryos bearing *Ubx* B, and 24B.GAL4 only (a and b), or 24B.GAL4 as well as UAS.Wg and/or UAS.Dpp, as indicated on the left (c–h), stained with antibody against lacZ (left-hand column) or *Ubx* (right-hand column; arrowheads point to anterior expansion of *Ubx* expression in d and h, and to posterior expansion in f and h). Note that ectopic Dpp prevents Wg-mediated repression posterior to the middle gut constriction (g and h). Symbols and orientations are as in Figure 1.

and expands *Ubx* expression towards the anterior (Figure 5d, arrowhead, compare with b), while Dpp alone expands *Ubx* expression towards the posterior (Figure 5f, arrowhead; see also Staehling-Hampton and Hoffmann, 1994). Remarkably, co-expression of both signals produces extensive ectopic *Ubx* expression, ranging from ~ps6 through to the end of the posterior midgut (~ps12) (Figure 5h). This demonstrates that Wg alone cannot stimulate *Ubx* transcription in the absence of detectable Dpp expression, and it confirms that high levels of Dpp prevent Wg from repressing *Ubx*.

High Wg represses its own expression

We wished to determine whether other Wg target genes in the visceral mesoderm might be repressed by this condition. One candidate gene is *wg* itself, as *wg* can be autoregulatory (Hooper, 1994; Yoffe *et al.*, 1994; Pai *et al.*, 1997). Furthermore, *wg* expression is expanded in *sgg* mutants (Figure 6c, compare with a), suggesting that *wg* may autostimulate itself in the midgut too.

Examining *wg* expression in the developing midgut by antibody staining, we found that the staining is initially detected posteriorly to the incipient middle gut constriction (Figure 6a), i.e. in ps8 (van den Heuvel *et al.*, 1989). Later, the staining straddles this constriction and is eventually seen mainly in ps7, anterior to the constriction (Figure 6b; note that the constriction itself does not move its position during development since it forms and remains at the boundary between ps7 and 8; Tremml and Bienz, 1989, and unpublished observations). Evidently, *wg* expression shifts towards the anterior with time, suggesting that *wg* may autorepress itself, starting from the posterior.

In an attempt to show this, we overexpressed a constitutive form of Arm (S10; Pai *et al.*, 1997) to produce high Wg pathway activity throughout the mesoderm. This condition is slightly milder than *sgg* loss-of-function

or Wg overexpression by 24B.GAL4 (our unpublished observations). As expected from the *sgg* mutants, we found that overexpressed S10 causes stimulated and ectopic Wg expression in several regions of the midgut, chiefly in ps6 and 7 as well as in ps9 (arrowheads in Figure 6e and f); however, we frequently observe a conspicuous staining gap in ps8, at the Wg source (Figure 6f, open triangle). In contrast, mesodermal Dpp expression produces a high level of Wg expression throughout ps8 (Figure 6d, arrowhead; see also Staehling-Hampton and Hoffmann, 1994). This strongly indicates that S10 represses *wg* in cells that experience high Wg and low Dpp signalling, but that it stimulates *wg* expression in cells that experience high levels of both signals. Therefore, our observations regarding *Ubx* also apply to *wg* in the midgut: high Wg levels repress *wg* and are prevented from doing so by concomitant high Dpp levels.

Discussion

We have provided evidence that Wg signalling acts at two thresholds in the visceral mesoderm to elicit two distinct transcriptional responses: low Wg levels stimulate transcription, whereas high Wg levels repress it. Interestingly, Wg-mediated repression of *Ubx* requires the Mad-binding sites in the *Ubx* midgut enhancer. Moreover, this repression requires a low level of Dpp signalling, but apparently not Mad itself, and is antagonized by high Dpp levels. This suggests a molecular model of how Wg-mediated repression might work, and how Dpp signalling impinges on it (Figure 7).

Two thresholds of Wg signalling in the visceral mesoderm

The following evidence indicates that Wg-mediated repression in the visceral mesoderm depends on the signalling

Fig. 6. Wg represses its own expression. Side views of 10–14 h-old embryos stained with antibody against Wg; (a and b) wild type; (c) *sgg*/*+* embryo derived from a *sgg* mutant germ line; (d) 24B.GAL4/UAS.Dpp; (e and f) 24B.GAL4/UAS.S10; symbols and orientations are as in Figure 1.

Fig. 7. Wg-mediated transcriptional repression in the embryonic midgut. Top, outline of the genetic interactions in the visceral mesoderm between the two homeotic genes (*Ubx* and *abd-A*) and the two primary signals (Wg and Dpp) that mediate endoderm induction (arrows, stimulation; barred lines, repression). Note that the stimulatory effects of Wg and Dpp on *Ubx* are known to be direct (i.e. conferred by dTCF and Mad, which are activated post-transcriptionally by these signals). *Ubx*, *dpp* and *wg* are also autostimulatory (not indicated). Below, putative profiles of Dpp and Wg signalling with respect to parasegmental (ps) positions and midgut constrictions (marked by 'V's). Bottom, presumed occupancies of the TCF- and Mad-binding sites in the various parasegmental regions that experience different levels of the two signals (ps5/6 junction, low Dpp, no Wg; ps7, high Dpp, low Wg; ps8, low Dpp, high Wg; ps9/10 junction, no Dpp, low Wg; only one TCF binding site, TCF B, is indicated). dTCF is thought to occupy the enhancer in the absence of signalling (Bienz, 1998), but is capable of stimulating transcription only if bound to Arm (whose stability is increased by Wg signalling). Mad is thought to occupy the enhancer strictly on Dpp signalling (as this promotes nuclear translocation of Mad). We propose that WR is a repressive Smad that occupies the enhancer as a consequence of low Dpp and high Wg signalling (see text). We further propose that WR and Mad compete for binding to the enhancer (perhaps as oligomers with their common partner Medea; see text). The area where we envisage this competition to be critical is at the ps7/8 border (arrow at bottom; see also text).

levels being high. First, while Wg-mediated stimulation of *Ubx* B is detectable even after short pulses of Wg overexpression, repression of *Ubx* B is seen only after more extensive and sustained Wg overexpression (see Figure 1). Repression does not appear to be linked to a particular time window of development. Secondly, Wg-induced repression correlates with proximity to the Wg source: the most sensitive cells for repression are those in and around the endogenous Wg source (in ps8; i.e. the cells that are expected to be stimulated the most by Wg). The same Wg overexpression conditions can

cause *Ubx* B stimulation in cells distal to the Wg source (in ps6 and ps10), but *Ubx* B repression in cells near this source (in ps8).

We previously have observed the same scenario in the endoderm, where low Wg levels stimulate *lab* transcription in cells distant from the Wg source while high Wg levels repress it in cells near this source (Hoppler and Bienz, 1995). In this cell layer, too, high Dpp antagonizes the repressive effect of Wg to some extent, although less efficiently than in the visceral mesoderm (unpublished observations). It seems that *lab* is more easily repressed

by Wg than is *Ubx*. Similarly, *Ubx* RP is more easily repressed by Wg than is *Ubx* B, which can only be repressed by artificially high Wg levels. These different sensitivities to Wg levels (and to antagonizing Dpp) probably reflect the different arrays of WRS-R and DRS motifs and their balances in the various target enhancers.

Thus, in both cell layers of the embryonic midgut, Wg signals at two distinct thresholds to elicit distinct transcriptional responses of Wg target genes. In the endoderm, each of these Wg thresholds specifies the differentiation of a unique cell type in the larval gut (Hoppler and Bienz, 1995). In the visceral mesoderm, low Wg stimulates *Ubx* transcription in ps7, while high levels repress *Ubx* in ps8 (Figure 7). What is the function of the two Wg thresholds in this cell layer of the midgut?

We believe that these signalling thresholds are critical in the reassessment process that takes place during the maintenance of the correct spatial expression pattern of *Ubx* throughout embryogenesis (reviewed in Bienz, 1997). Wg-mediated stimulation of *Ubx* is part of a positive paracrine feedback loop ensuring coordinated expression of *Ubx* in the ps7 cell group (Thüringer and Bienz, 1993). In contrast, the Wg-mediated repression should prevent *Ubx* expression from spreading towards posterior and into the Wg source, thus delineating sharply the posterior expression limit of *Ubx*. A morphological consequence of this process of delimiting *Ubx* posteriorly may be the formation of the middle gut constriction, or more generally the subdivision of the visceral mesoderm into distinct sections. We do not know whether this subdivision is critical for subsequent cellular differentiation of the visceral musculature. Regardless of this, we presume that the precise definition of the group of *Ubx*-expressing cells is important for the process of endoderm induction which is initiated by *Ubx*.

Recall that the homeotic gene *abd-A* also represses *Ubx* in the posterior midgut (Biernz and Tremml, 1988; Figure 7). Some of this repression may be directly due to Abd-A protein binding to *Ubx* cis-regulatory DNA, since it has been reported that naturally occurring Abd-A binding sites can act as repressive DNA elements (Appel and Sakonju, 1993). If so, Abd-A-mediated repression would be distinct from and in parallel to repression by high Wg, since the sequence of the WRS-R bears no resemblance to Abd-A binding sites (see Appel and Sakonju, 1993). It thus appears that *abd-A* activates expression of a signalling gene, *wg*, which may help Abd-A to repress *Ubx* in cells that express *abd-A*, but which stimulates *Ubx* expression in cells further away that do not express *abd-A* (Figure 7). Abd-A may be incapable of repressing *Ubx* by itself, and may thus rely on the help of *wg* to do this. This putative cooperation between the Abd-A repressor and Wg signalling could provide precision and reliability in the repressive process which sharply delineates the posterior expression limit of *Ubx*.

dpp is required for Wg-mediated repression

As mentioned above, Dpp and Wg signalling synergize in the visceral mesoderm to stimulate *Ubx* transcription, targeting distinct, albeit adjacent, response sequences in the *Ubx* midgut enhancer. So, efficient stimulation of *Ubx* transcription by Wg depends on *dpp*. Here, we have shown that Wg-mediated repression also depends on *dpp*, but,

remarkably in this case, the response sequence for Wg-mediated repression within the *Ubx* enhancer coincides with that for Dpp-mediated stimulation. Indeed, the WRS-R/DRS functions in two antipodal responses: it mediates efficient transcriptional stimulation when the signalling levels of Dpp are high and those of Wg are low, but it is also required for transcriptional repression when the Wg signalling levels are high and those of Dpp are low (Figure 7). This raised the possibility that the same factor may confer the two antipodal responses. However, this is unlikely to be the case since Mad itself, which binds to the DRS to mediate the positive response to Dpp (Newfeld *et al.*, 1996; Kim *et al.*, 1997), is apparently not required for the Wg-mediated repression.

We thus propose that the two antipodal responses are conferred by two distinct factors, by Mad and by a hypothetical protein WR (Figure 7). We further propose that WR is a Mad-related protein, i.e. a Smad, since WR acts through Mad-binding sites and since its function as a repressor depends on *dpp*. We envisage that WR, like Mad itself and other Smads, is activated by Dpp signalling through phosphorylation by ligand-bound membrane receptors, an event that promotes their subsequent translocation to the nucleus (Massagué *et al.*, 1997). In this scenario, *dpp* enables WR (which also needs to be activated by high Wg signalling, see below) to occupy the Mad-binding sites within the *Ubx* enhancer. Once bound to this enhancer, WR dominantly represses *Ubx* transcription, overriding the activating function of Arm-dTCF and other transcriptional activators bound to the same enhancer.

How is WR's repressor function activated by high Wg levels? We presume that high Wg signalling regulates, directly or indirectly, the availability of WR as an enhancer-binding protein: either high Wg signalling controls a post-transcriptional event (e.g. it may promote WR's association with Armadillo, or WR's translocation into the nucleus), or it simply activates transcription of the WR gene. We favour the latter possibility of indirect regulation, which involves transcriptional coupling, because it accommodates readily the dependence of Wg-mediated repression on *arm* and *dTCF* (Figure 2). Whatever the case, we would like to emphasize that high Wg signalling controls the activity of the protein WR, possibly a Smad, which also requires Dpp signalling. Thus, WR is a common target for two signalling pathways and represents a point of convergence between them.

Our model readily explains how high Dpp levels antagonize WR, namely by promoting maximal levels of nuclear Mad which now competes with WR for binding to the *Ubx* enhancer. The outcome of this competition is the transcriptional activation or repression of target genes, depending on the prevalence of Mad or WR (Figure 7). This may illustrate a general principle, namely that the response sequence for the positive effect of one signal is also the response sequence for the negative effect of an antagonistic signal. Such a layout provides a sharp flipping of the response from positive to negative in an area where cells are experiencing increasingly more of one signal and increasingly less of the antagonizing one (Figure 7, arrow indicating competition).

Recently, it was reported that Medea is the Smad4 homolog which is known to be the common oligomerization partner for pathway-specific Smads (Das *et al.*, 1998;

Hudson *et al.*, 1998; Inoue *et al.*, 1998; Wisotzkey *et al.*, 1998). Furthermore, Medea binds to the same DNA sequences as Mad (Xu *et al.*, 1998). This raises the possibility that Medea is an oligomerization partner of WR and, while Medea together with Mad is expected to activate transcription, together with WR it may repress transcription. A precedent for this scenario is the Myc/Mad/Max system, in which Mad (a bHLH protein which happens to be named the same as the Dpp transducer Mad) is a common dimerization partner for either Myc, a transcriptional activator, or Max, a transcriptional repressor (Ayer *et al.*, 1993).

Finally, recall that, in addition to antagonism, there is also synergy between Wg and Dpp in the embryonic midgut (Thüringer *et al.*, 1993; this work). This synergy apparently results from cooperation between the nuclear target factors activated by the two signals, i.e. between Arm-dTCF and Mad/CRE-binding proteins (Eresh *et al.*, 1997; Riese *et al.*, 1997; Szüts *et al.*, 1998). Other examples of apparent synergy between Wg and Dpp are the leg and wing imaginal discs, where these signals act together in central disc regions to stimulate expression of homeobox genes (Campbell *et al.*, 1993; Diaz-Benjumea *et al.*, 1994). But the two signals also antagonize each other in leg discs (Brook and Cohen, 1996; Jiang and Struhl, 1996; Penton and Hoffmann, 1996; Theisen *et al.*, 1996), as well as in eye discs (Chanut and Heberlein, 1997; Dominguez and Hafen, 1997). Although it is conceivable that the developmental context determines the synergy or antagonism between Dpp and Wg, the situation in the midgut suggests that the decisive factor in each case may be the levels of signalling.

Wg represses its own expression

It is interesting that Wg signalling can repress its own expression when signalling levels reach a critically high level. This indicates a negative feedback loop which could account for two observations. First, Wg signalling shifts its own expression towards the anterior over time (Figure 6). We do not know at present whether this shift has any biological significance. Secondly, Wg has the potential of switching itself off over time. This is actually observed, as Wg expression becomes undetectable by the end of embryogenesis. Clearly, this negative feedback loop of Wg is capable of changing the Wg signalling profile as development proceeds.

There are negative feedback loops of other signalling pathways in *Drosophila*. For example, the epidermal growth factor (EGF) receptor inhibits itself eventually, after signalling has reached a critical level, by switching on expression of an inhibitory ligand, Argos (Golembo *et al.*, 1996; Freeman, 1997). In the ovary, this negative feedback loop causes splitting of a single signalling peak into twin peaks (Wasserman and Freeman, 1998). Furthermore, Hedgehog signalling in the eye imaginal disc is repressive at high Hedgehog levels, but stimulatory in cells further away from the signalling source that experience lower Hedgehog levels (M.Dominguez, in preparation). Perhaps such 'hard-wired' negative feedback loops of signalling pathways are fairly universal, stopping these pathways from escalating out of control. If so, this would be akin to feedback inhibition of metabolic pathways, which provides homeostatic control.

Materials and methods

Fly strains

The following mutant alleles were used: *wg^{cx4}* (Baker, 1987); *dpp^{s4}* (Immerglück *et al.*, 1990); *dTCF²* (van de Wetering *et al.*, 1997); *arm^{H8.6}* (Peifer and Wieschaus, 1990); *abd-A^{M1}* (Casanova *et al.*, 1987); and *mad¹²*, *Df(2L)JS17* [an apparent *mad* null allele and a deficiency uncovering *mad*, respectively; Sekelsky *et al.* (1995); *mad* mutant embryos were generated from these two stocks as in Newfeld *et al.* (1996)]. *sgg* mutant embryos were generated from mutant *zw3^{M11}* germ lines as described previously (Yu *et al.*, 1996).

Wg was ubiquitously overexpressed with the hs-wg transposon described earlier (Noordermeer *et al.*, 1992). The following GAL4 driver and producer lines were used: 24B.GAL4 (Brand and Perrimon, 1993), UAS.Wg (Lawrence *et al.*, 1996), UAS.Dpp (Stachling-Hampton and Hoffmann, 1994) and UAS.S10 (Pai *et al.*, 1997). To overexpress Wg in *wg* and *dpp* mutant embryos, a recombinant chromosome bearing both 24B.GAL4 and *Ubx* B was used (provided by David Szüts).

The following lacZ reporter constructs were used: RP, Bhz, ΔD, ΔDI and ΔDII (Thüringer *et al.*, 1993); B5, BC (Eresh *et al.*, 1997); B4, BG and BL (Riese *et al.*, 1997); BM1, BM2 and BC2 (Szüts *et al.*, 1998). The following additional mutant enhancers were generated by the introduction of base substitutions (indicated by small letters; all sequences below are in the antisense orientation) into *Ubx* B as described (Eresh *et al.*, 1997; Riese, 1997): TTCGAGATCCAgggcccTCGTGGCCCC for B3; TAAAGCAGacgaTcGGCAGCTGG for B6; ATTTGGCAGCcGatcGCGCCGGC and GGTGGAAAGCAttcgAATCAGGGGGG for BE; CTGACGCCAGaggcccACATCAAACGG and CTAATCATAGaC-CgAtcgAGATACATTGCG for B4R8. For each mutant enhancer construct, several independent transformant lines were isolated and tested for their response to Wg.

We evaluated the resulting lacZ staining patterns chiefly by comparing the staining intensities posterior (ps8–10) versus anterior (ps2–7) to the middle gut constriction in 24B.GAL4/UAS.Wg embryos, but also by taking into account the staining levels in control embryos. The following four groups of (mutant) enhancers were classified, based on the levels of lacZ staining posterior to the middle gut constriction of 24B.GAL4/UAS.Wg embryos: (i) *Ubx* B, B3, B4, B4R8, BG, BL, BC2 and ΔDII, no staining; (ii) BE and B6, some staining; (iii) BC, B5 and BM1, moderate levels of staining, but less than anterior to the constriction; (iv) ΔD, ΔDI and BM2, same staining levels anterior and posterior to the constriction. We found that whether a mutant enhancer is repressible by high Wg is not necessarily determined by whether it can be stimulated by Dpp. For example, B5 and BM1 still respond strongly to Dpp in the anterior midgut, while BC completely lacks such a response, yet they are all similarly expanded posteriorly in response to high Wg. Finally, it is noteworthy that, unlike ΔD and ΔDI, BM2 shows staining outside ps3–10, probably because it contains a fully functional WRS. BM2 also shows some repression around the middle gut constriction of the 24B.GAL4/UAS.Wg embryos, i.e. in cells which experience high levels of endogenous Dpp, suggesting that this mutant enhancer may show a negative response to Dpp.

B3B4 was generated by integrating the residues mutated in B3 and B4 into a single construct. Multiple transgenic lines were isolated, but none of these showed any lacZ staining in any part of the embryo, even after overexpression of Wg or Dpp by 24B.GAL4. The presence of intact mutant constructs in the different transgenic lines was confirmed by sequencing of PCR-amplified fragments spanning the mutated enhancer residues and the 5' end of the lacZ reporter gene.

DNA binding of mouse LEF-1 to TCF A and B was carried out as described previously (Riese *et al.*, 1997).

Heat-shock regimes and phenotypic analysis

Agarose plates containing embryos (collected for 4 h at 25°C and aged appropriately) were immersed in a 37°C waterbath for 20 min and subsequently aged for 2 h at 25°C prior to the next heat shock or to fixation. *Ubx* B repression in ps8 was observed when 10- to 14-h-old embryos were subjected to a single heat shock of 20 min, as reported (Thüringer *et al.*, 1993; Riese *et al.*, 1997), but not if 8- to 12-h-old embryos were subjected to the same heat shock and aged for 4 h prior to fixation. If a further heat shock was applied 2 h later, more extensive repression was observed. If embryos were subjected to three heat shocks at 2 h intervals, repression was maximal (i.e. as extensive as that seen in 24B.GAL4/UAS.Wg embryos). In contrast, four consecutive heat-shocks of 5 min (spaced by 2 h intervals) applied to 4- to 8-h-old embryos did not produce any repression.

Staining of embryos was performed as described (Yu *et al.*, 1996).

The following antibodies were used: anti-Ubx (White and Wilcox, 1984), anti-Wg (Brook and Cohen, 1996) and anti-lacZ (Promega). Mutant embryos were identified by their gut phenotypes, by changes in reporter expression and/or by lacZ staining due to 'blue' balancer chromosomes.

Acknowledgements

We thank Jean-Paul Vincent, Mark Peifer, Marek Mlodzik and David Szüts for fly strains; Steve Cohen for the Wingless antibody, and Matthew Freeman and Maria Dominguez for comments on the manuscript. X.Y. is supported by a studentship from Trinity College, Cambridge. J.R. was a Medical Research Council student supported by the Boehringer Ingelheim Fonds.

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Received July 8, 1998; revised October 6, 1998;
accepted October 7, 1998