Coordinate regulation of downstream genes by *extradenticle* and the homeotic selector proteins

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Communicated by W.Gehring

Mutations in the *Drosophila* gene *extradenticle* (*exd*), a homologue of the human proto-oncogene *pbx1*, cause homeotic transformations by altering the morphological consequences of homeotic selector gene activity. *exd* has been proposed to act by contributing to the specificity of selector homeodomain proteins for their downstream targets. Here we show that *exd* is indeed required for the appropriate regulation of at least some of these target genes. Expression patterns of *wingless*, *teashirt* and *decapentaplegic* (*dpp*) are altered in the embryonic midgut of embryos lacking *exd*, while the expression of their respective regulators (*abd-A*, *Antp* and *Ubx*) remains normal. Co-regulation of *dpp* by *exd* and *Ubx* was investigated in greater detail by examining the expression of reporter constructs in *exd* embryos. These experiments not only define *dpp* regulatory regions responsive to *exd*, but also distinguish two functions of *exd* in the regulation of *dpp*. *exd* acts with *Ubx* to activate *dpp* expression in parasegment 7 (PS7), via a minimal visceral mesoderm enhancer, and *exd* represses *dpp* expression anterior to PS7. We show that even when *Ubx* is ubiquitously expressed at high levels in *exd* embryos, *Ubx* is incapable of activating *dpp* enhancer expression. Thus, *exd* is an indispensable component in target gene regulation by the homeotic selector proteins.

Key words: *decapentaplegic/extradenticle/homeodomain/target specificity*

Introduction

The homeotic selector genes are the key regulators that distinguish different body regions along the anterior–posterior axis during the development of an organism (reviewed by McGinnis and Krumlauf, 1992). These genes are grouped in the Antennapedia and bithorax (HOM) complexes in flies and in the Hox gene clusters in vertebrates. Mutations that eliminate or alter expression of these genes result in transformation of the affected segments toward the morphology of other segments. The proteins encoded by these HOM/Hox genes share a motif known as the homeodomain, also found in a variety of other transcription factors (reviewed by Scott *et al.*, 1989). The homeodomain is responsible for sequence-specific DNA recognition (reviewed by Hayashi and Scott, 1990). Consistent with their ability to bind DNA, these proteins act as transcriptional activators and repressors (Levine

and Hoey, 1988; Thali *et al.*, 1988; Krasnow *et al.*, 1989; Winslow *et al.*, 1989; Johnson and Krasnow, 1990) and thus presumably specify different developmental fates by controlling the expression of distinct sets of downstream genes. However, because the homeodomains of these selector proteins are very similar to one another and because different homeodomains can bind similar DNA sequences with similar affinities *in vitro* (Desplan *et al.*, 1988; Hoey and Levine, 1988; Percival-Smith *et al.*, 1990), it remains unclear how these selector proteins achieve distinct regulation of downstream target genes *in vivo*. It has been suggested that distinct DNA binding specificity *in vivo* could be afforded by interactions with additional proteins (Hayashi and Scott, 1990). Such proteins might alter the specific DNA contacts made by selector proteins, thereby allowing only very specific sequences to be bound, or they might be involved in cooperative binding, thereby increasing the affinity of selector proteins for particular sites. Indeed, other non-selector homeodomain proteins are involved in protein–protein interactions with non-homeodomain cofactors (Grueneberg *et al.*, 1992; Lai *et al.*, 1992; Pomerantz *et al.*, 1992; Smith and Johnson, 1992), while others bind to DNA cooperatively with other homeodomain proteins as homo- or heterodimers (Goutte and Johnson, 1993; Wilson *et al.*, 1993; Xue *et al.*, 1993). However, a mechanism by which homeotic selector proteins increase specificity for target sites remains to be elucidated.

The *Drosophila* gene *extradenticle* (*exd*) is a good candidate for encoding a protein which facilitates the recognition of sites within target genes by the homeotic selector proteins. The *exd* protein (EXD) contains a homeodomain and shares extensive sequence identity (71%) with the human proto-oncoprotein Pbx1 and with additional members of a related protein family, namely human Pbx2 and Pbx3 and *Caenorhabditis elegans* *ceh-20* (Bürglin and Ruvkun, 1992; Flegel *et al.*, 1993; Rauskolb *et al.*, 1993). *exd* is expressed throughout development and in the multiple tissues that require homeotic selector gene activity. Mutations in *exd* result in homeotic transformations, although in contrast to other known homeotic mutations they do not appear to affect the expression of the homeotic selector genes (Peifer and Wieschaus, 1990). Thus, the effects of *exd* mutations are not due to misregulation of homeotic selector gene expression. Moreover, *exd* does not act as a downstream target gene, as its function does not depend on regulation by the homeotic selector genes. *exd* is supplied to the embryo both maternally and zygotically. If the maternal *exd* is increased it can rescue the homeotic transformations due to loss of zygotic *exd* (Wieschaus and Noell, 1986). Together these results indicate that *exd* acts in parallel to the homeotic selector proteins in the determination of segmental morphologies by altering the morphological consequences of their activ-

ity. To explain these observations, we postulated that *exd* could alter the outcome of homeotic gene activity by affecting the manner in which these homeodomain proteins bind to specific DNA sequences in the regulation of downstream gene expression (Peifer and Wieschaus, 1990; Rauskolb *et al.*, 1993). Since EXD itself appears to be a DNA binding transcription factor, an attractive possibility is that EXD and the homeotic selector proteins act together to coordinately regulate the same set of target genes. In this case, targets of the homeotic selector proteins would then also respond to *exd*. Alternatively, *exd* could act in a parallel pathway and affect morphology by regulating a set of target genes distinct from those regulated by the homeotics. In this case, the expression of homeotic target genes would not be altered by the absence of *exd*.

To address these questions we have investigated the effects of *exd* mutations on the expression of homeotic target genes in the embryonic midgut. The midgut has proven to be a valuable system for studying the regulation of downstream targets by the homeotic selector genes. It is a relatively simple structure composed of two cell layers, an inner layer of endodermal cells and an external layer of visceral mesoderm cells. The homeotic selector genes, *Antennapedia* (*Antp*), *Ultrabithorax* (*Ubx*) and *abdominal-A* (*abd-A*), are expressed in distinct units, termed parasegments (PS), in the visceral mesoderm (Bienz and Tremml, 1988; Bienz *et al.*, 1988; Tremml and Bienz, 1989; Reuter and Scott, 1990). They control essential aspects of midgut morphogenesis, including the constrictions that subdivide the midgut into compartments. Most importantly, a number of genes whose expression is regulated by these homeotic selectors have been identified in the visceral mesoderm (Immerglück *et al.*, 1990; Reuter *et al.*, 1990). In the case of the target gene *decapentaplegic* (*dpp*), it has been demonstrated that the selector protein UBX binds directly to *dpp* regulatory sequences *in vivo* to promote expression (Capovilla *et al.*, 1994). Like the homeotic selector genes, these downstream genes are required for discrete aspects of midgut morphogenesis (Immerglück *et al.*, 1990; Panganiban *et al.*, 1990; Reuter *et al.*, 1990). Here we demonstrate that three targets of the homeotic selector genes, *dpp*, *wingless* and *teashirt*, are also regulated by *exd*. Furthermore, by using reporter constructs in which *dpp* regulatory sequences are fused to *LacZ* (Capovilla *et al.*, 1994), we localize *exd* responsive sites within the *dpp* regulatory region and show that *exd* both positively and negatively regulates *dpp* expression. We find that *exd* and *Ubx* positively regulate *dpp* via the same minimal visceral mesoderm enhancer and, importantly, that *Ubx* requires *exd* in order to activate this enhancer.

Results

exd positively regulates the expression of the homeotic target genes *wingless* and *teashirt*

In the ectoderm, *exd* alters segmental identities by acting in parallel to the homeotic selector genes. In order to confirm that the visceral mesoderm could be used as a model tissue for investigating the effects of *exd* on homeotic target genes, we first examined selector gene expression in *exd* mutant embryos. This analysis indicated that the homeotic selector genes *Antp*, *Ubx* and *abd-A* are

expressed normally in the visceral mesoderm of embryos lacking all *exd* product (data not shown), demonstrating that *exd* does not regulate their expression. It was thus possible to test whether or not *exd* acts in parallel to these homeotic selector proteins by regulating the expression of the same downstream genes. In other words, if *exd* acts by affecting the ability of these homeodomain proteins to regulate their normal target genes, then in the absence of *exd* homeotic target gene expression should be altered.

One homeotic target gene expressed in the visceral mesoderm is *wingless* (*wg*). *wg* is expressed in PS8 of the visceral mesoderm (Figure 1A; van den Heuvel *et al.*, 1989), where it is required for the formation of the second midgut constriction (Immerglück *et al.*, 1990). Its expression is controlled by *abd-A*: in *abd-A* mutants, *wg* is not detected in the visceral mesoderm (Immerglück *et al.*, 1990; Reuter *et al.*, 1990). To test whether the expression of this homeotic target gene is dependent upon *exd*, we examined *wg* expression in embryos lacking both maternal and zygotic *exd* product. The absence of *wg* expression from the visceral mesoderm is readily apparent in these embryos lacking all *exd* product (Figure 1B). *wg* expression is, however, observed in embryos lacking only zygotic *exd* (data not shown), indicating that uniform maternal *exd* product is sufficient for *wg* expression in PS8. These results establish that both *exd* and *abd-A* are required for positive regulation of the target gene *wg*.

The expression of the *Antp* target gene, *teashirt* (*tsh*), was also examined. *tsh* is expressed in two domains in the visceral mesoderm, in PS5/6 and in PS8 (Figure 1C; L.Mathies and M.Scott, personal communication), and is required for the formation of both the first and second midgut constrictions. *Antp* is required for positive regulation of *tsh* expression in PS5 and PS6. In *Antp* mutants this domain of *tsh* expression is nearly absent (Figure 1E; L.Mathies and M.Scott, personal communication), while the more posterior domain of expression remains unaffected. In embryos lacking *exd*, *tsh* expression is absent from the entire midgut (Figure 1D). Thus, in PS5 and PS6, both *exd* and *Antp* are required to positively regulate *tsh*. Given that *wg* mutants lack *tsh* expression in PS8 (L.Mathies and M.Scott, personal communication), the absence of *tsh* expression in this domain of *exd* mutants could be a secondary consequence of the lack of *wg* expression in these embryos.

exd is required for the correct expression of *dpp* in the visceral mesoderm

The best characterized and only proven direct homeotic target gene is *dpp* (Capovilla *et al.*, 1994). In wild-type embryos, *dpp* is highly expressed in the anlagen of the gastric caeca and in PS7, the site of the second midgut constriction (Figure 2A; Immerglück *et al.*, 1990; Panganiban *et al.*, 1990; Hursh *et al.*, 1993). This expression is essential for midgut morphogenesis: *dpp* mutations that eliminate midgut expression disrupt the formation of both the gastric caeca and the second constriction. The homeotic selector genes *Ubx* and *abd-A* regulate *dpp* expression in the visceral mesoderm; *Ubx* positively regulates *dpp* in PS7 and *abd-A* negatively regulates *dpp* in more posterior parasegments (Immerglück *et al.*, 1990; Reuter *et al.*, 1990).

In embryos lacking *exd*, ectopic *dpp* expression is

Fig. 1. *exd* positively regulates the homeotic target genes *wg* and *tsh*. Expression of these targets was detected by *in situ* hybridization. (A) In wild-type embryos, *wg* is expressed in PS8 of the visceral mesoderm (arrowhead). (B) In embryos lacking both maternal and zygotic *exd*, *wg* is not expressed in PS8. (C) In the wild-type, *tsh* is expressed in PS5 and PS6 (arrowhead) and also in PS8 (arrow). (D) In embryos lacking *exd*, expression of *tsh* is absent from the entire visceral mesoderm. Also, epidermal *tsh* expression is diminished in these embryos. (E) In *Antp* mutant embryos, expression of *tsh* is greatly reduced in PS5 and PS6 (arrowhead), while expression in PS8 (arrow) remains unaffected. This embryo was stained longer than the one in (C): the PS8 expression is not actually elevated relative to wild-type.

observed in the visceral mesoderm anterior to PS7, while some *dpp* expression is lost within PS7 (Figure 2B, cf. 2A, Figure 3). Thus, *exd* is required for the appropriate regulation of this homeotic target gene. This alteration in *dpp* expression requires the removal of both maternal and zygotic *exd* product. In the presence of either maternal or zygotic *exd* alone, *dpp* is expressed normally (Figure 2A, data not shown). Uniform maternal *exd* product is thus sufficient for homeotic target gene expression. We first addressed what factor might be responsible for the ectopic expression of *dpp* in the absence of *exd*. Although *Ubx* expression appears to be normal in embryos completely lacking *exd*, it was possible that *Ubx* could be expressed in the anterior midgut at levels too low to be detected by antibody staining yet high enough to activate *dpp* expression. We therefore examined embryos lacking both *exd* and *Ubx* and found that in such mutant embryos *dpp* is still expressed ectopically (Figure 2D). Hence, the *dpp* expression observed in the anterior midgut of embryos lacking *exd* is not dependent on *Ubx*. Because the homeotic gene *Antp* is expressed in the region of ectopic *dpp* and phenotypic analysis of *exd* mutants had suggested that selector proteins could acquire the activity of other selector proteins, we considered the possibility that in the absence of *exd*, *Antp* might activate *dpp*. However, examination of *dpp* expression in embryos lacking both *exd* and *Antp* ruled out this possibility. In the absence of *Antp* alone, only a slight change in the spatial pattern of *dpp* transcripts is detected (Figure 2E). In contrast, in *exd Antp* double mutant embryos *dpp* transcripts are detected in the anterior

midgut in the same pattern as observed in embryos mutant for *exd* alone (Figure 2F, cf. 2B). Together these results demonstrate that *exd* is required to repress *dpp* expression in the anterior midgut and that the ectopic *dpp* expression observed in the absence of *exd* is not mediated by either *Antp* or *Ubx*, but rather by some unknown factor.

Analysis of the effect of *exd* on the *Ubx* controlled PS7 expression of *dpp* is complicated by the *Ubx*-independent ectopic expression of *dpp* in the anterior midgut. However, a closer examination of *exd* embryos shows that *dpp* expression in PS7 is patchy and not all cells express *dpp*. Double staining of *exd* mutant embryos for *Ubx* and *dpp* confirms that there are cells that express *Ubx* but do not transcribe *dpp* (Figure 3B and C). Normally, in wild-type embryos, all cells expressing *Ubx* simultaneously express *dpp* (Figure 3A; Reuter *et al.*, 1990). Thus, in the absence of *exd* the ability of *Ubx* to positively regulate its target gene *dpp* appears to be compromised.

In *exd* embryos *dpp* is not ectopically expressed in the posterior end of the midgut, the domain in which *abd-A* is expressed. This suggests that in the absence of *exd*, *abd-A* can still repress *dpp*. This was further supported by experiments in which *Ubx* was expressed throughout the midgut by heat-shocking *exd* mutant embryos carrying *HS:Ubx*. In wild-type embryos under these conditions, *dpp* expression does not extend into the posterior end of the midgut, due to repression by *abd-A* (Figure 2G; Reuter *et al.*, 1990). Likewise, embryos mutant for *exd* and carrying *HS:Ubx*, when heat-shocked, do not express *dpp* posterior to PS7 (Figure 2H). Because *dpp* expression is

Fig. 2. Expression of the homeotic target gene *dpp* in embryos lacking *exd*. *In situ* hybridization was used to detect *dpp* transcripts in the visceral mesoderm of various mutant and transformant embryos. (A) In wild-type embryos, *dpp* is expressed at high levels in both PS7 (arrowhead) and in the anlagen of the gastric caeca (arrow). These embryos lack maternal *exd* product, but express wild-type zygotic *exd*. (B) In embryos lacking both maternal and zygotic *exd*, expression of *dpp* extends from PS7 to the anterior end of the midgut. Note that transcript levels are not uniform and expression in some regions appears patchy. (C) In *Ubx* mutants, expression of *dpp* in PS7 is reduced (arrowhead), while expression in the anlagen of the gastric caeca is unaffected. (D) In *exd Ubx* mutant embryos from *exd* germline mosaic females, the ectopic anterior expansion of *dpp* is still observed. (E) *Antp* mutant embryos have only a slight alteration in the expression of *dpp*. (F) *exd Antp* mutant embryos from *exd* germline mosaic females still ectopically express *dpp*. (G) A heat-shocked wild-type embryo carrying *HS:Ubx* ectopically expresses *dpp* in PS7 and more anterior parasegments. The expression does not extend posterior to PS7 because of repression by *abd-A*. (H) A heat-shocked embryo carrying *HS:Ubx* and lacking *exd* shows, like the wild-type in (G), expression of *dpp* in PS7 and parasegments anterior, but not posterior, to PS7.

not observed in the posterior region of the midgut, *abd-A* must still be capable of repressing *dpp* even in the absence of *exd*.

***exd* and *Ubx* regulate *dpp* via the same visceral mesoderm enhancer**

To further understand the role of *exd* in the regulation of *dpp* expression, we mapped *exd* responsive elements within the *dpp* regulatory region. Sequences necessary for proper expression of *dpp* in the embryonic visceral mesoderm have been localized to the *shortvein* cis-regulatory region of *dpp* (Immerglück *et al.*, 1990; Panganiban *et al.*, 1990). Constructs that fuse DNA fragments derived from the *shortvein* region to heterologous promoters driving β -galactosidase expression recapitulate many

aspects of the endogenous *dpp* expression pattern in the visceral mesoderm (Hursh *et al.*, 1993; Masucci and Hoffmann, 1993; Capovilla *et al.*, 1994). We have made use of flies carrying various β -galactosidase reporter constructs to localize sequences required for the regulation of *dpp* by *exd*.

Reporter constructs containing either a 4 kb fragment (*dpp4000lacZ*) or a smaller 674 bp subfragment (*dpp674lacZ*) from the *dpp* regulatory region express β -galactosidase in both the anlagen of the gastric caeca and in PS7 of wild-type embryos (Figure 4A and C; Capovilla *et al.*, 1994). The spatial restriction of this expression is indistinguishable from *dpp* transcript accumulation. In *exd* mutant embryos carrying these constructs, β -galactosidase is ectopically expressed anterior to PS7 (Figure 4B and

Fig. 3. Not all UBX-expressing cells transcribe *dpp* in embryos lacking *exd*. Antibodies were used to detect *Ubx* protein (UBX; in brown), while *in situ* hybridization using digoxigenin-labelled cDNA probe was used to detect *dpp* transcripts (in blue). (A) In the wild-type, all UBX-expressing cells in the visceral mesoderm simultaneously express *dpp*, as evidenced by the overlap in brown and blue staining (arrowhead). (B) In embryos lacking *exd*, although some UBX-expressing cells do transcribe *dpp*, others do not, as there are cells in the visceral mesoderm stained brown but not blue (arrowhead). (C) A higher magnification of (B) clearly showing that while some cells express UBX and *dpp* (arrowhead) in embryos lacking *exd*, not all UBX-expressing cells transcribe *dpp* (arrow).

D). These reporter genes thus mimic the normal regulation of endogenous *dpp* by *exd*. These results demonstrate that *exd* responsive elements are present in 674 bp of the *dpp* regulatory region and that *exd* mediates repression of *dpp* via these sites. There is some difference in levels of ectopic β -galactosidase expression between *dpp4000lacZ* and *dpp674lacZ* (Figure 4B and D); this presumably reflects a loss of enhancer elements in the smaller reporter construct.

exd responsive sequences were further localized by taking advantage of a 303 bp subfragment of *dpp674* (Capovilla *et al.*, 1994). Normally, in wild-type embryos, expression of this reporter construct, *dpp303lacZ*, is first detected in PS7 at germband retracted stages. Weak expression in the anlagen of the gastric caeca appears later. *Ubx* is required for the positive regulation of *dpp303lacZ* in PS7; in *Ubx* mutants, expression of this reporter construct is greatly reduced (Capovilla *et al.*, 1994). We see a similar reduction in expression of *dpp303lacZ* in *exd* embryos. Expression is weak and in only a few cells of PS7 at a stage when, in the wild-type, β -galactosidase

expression is clearly detected in all visceral mesoderm cells of PS7 (Figure 4E and F). These results thus extend the observation that *dpp* transcripts are not detected in some UBX-expressing cells of *exd* mutant embryos, confirming that *exd* mutations do, in fact, compromise the ability of *Ubx* to regulate target gene expression. In addition, they show that *exd* and *Ubx* can both positively regulate *dpp* through the same 303 bp visceral mesoderm enhancer. The observation that expression of *dpp303lacZ* is effectively absent in *exd* embryos also indicates that this construct lacks those sequences mediating ectopic *dpp* expression in the anterior visceral mesoderm.

A further demonstration that it is the action of UBX on this minimal enhancer that is specifically affected by *exd* comes from experiments making use of *HS:Ubx*. Like the endogenous *dpp* gene, *dpp303lacZ* is ectopically expressed in the anterior region of the midgut in embryos carrying *HS:Ubx* following heat-shock (Figure 4G). In contrast, in *exd HS:Ubx* embryos expression of the *dpp303lacZ* reporter construct is not detected (Figure 4H). These results indicate that it is not the levels of UBX which are affected by the absence of *exd*, but rather the activity of UBX itself. In the absence of *exd*, UBX is rendered non-functional in its ability to activate *dpp303lacZ* expression. These results also indicate that high levels of UBX cannot compensate for the loss of *exd* product.

Midgut morphogenesis is abnormal in *exd* mutant embryos

The embryonic midgut undergoes morphological changes that are dependent on the expression of the homeotic genes and their targets (Bienz and Tremml, 1988; Tremml and Bienz, 1989; Immerglück *et al.*, 1990; Panganiban *et al.*, 1990; Reuter and Scott, 1990; Hursh *et al.*, 1993). Since target gene expression is altered in *exd* embryos, we investigated whether consequent gut morphogenesis was affected. To examine the effects of *exd* mutations on the morphogenesis of the embryonic midgut, embryos lacking maternal and zygotic *exd* product were stained for muscle myosin, which is expressed by the visceral mesoderm cells (Reuter and Scott, 1990). This procedure allows for easy visualization of the gastric caeca and gut constrictions. Up to stage 14, the midgut of *exd* embryos is normal (Figure 5A–D). Subsequent gut morphogenesis is aberrant; the gastric caeca and the first and second constrictions never form in embryos lacking *exd* (Figure 5F, cf. 5E). It is difficult to assess whether the third constriction forms, as the abnormal gut in these embryos produces ectopic folds which obscure proper visualization of the third constriction.

These results are consistent with the altered target gene expression in *exd* mutants. *Scr* is normally required for gastric caeca development (Reuter and Scott, 1990); in *exd* mutant embryos *Scr* is not expressed (data not shown) and the gastric caeca fail to form. The formation of the first constriction requires *tsh* function (L.Mathies and M.Scott, personal communication); in *exd* mutants *tsh* is not expressed and the first constriction does not form. In addition, formation of the second constriction requires the activity of *dpp*, *wg* and *tsh* (Immerglück *et al.*, 1990; Panganiban *et al.*, 1990; Reuter *et al.*, 1990; L.Mathies and M.Scott, personal communication). In *exd* mutants these genes are not expressed appropriately and the second

Fig. 4. Expression of *dpp4000lacZ*, *dpp674lacZ* and *dpp303lacZ* in *exd* mutant embryos. Expression of these reporter constructs was detected by using antibodies directed against β -galactosidase. (A) *dpp4000lacZ* in wild-type embryos is expressed in PS7 and in the anlagen of the gastric caeca, like the endogenous *dpp* gene (cf. Figure 2A). (B) *dpp4000lacZ* in embryos lacking both maternal and zygotic *exd* is expressed ectopically anterior to PS7. This expression mimics the expression of *dpp* in these embryos (cf. Figure 2B), but levels are higher and more uniform. (C) *dpp674lacZ* expression in wild-type embryos. (D) *dpp674lacZ* expression in embryos lacking *exd*. This reporter construct, like *dpp4000lacZ*, is expressed ectopically in the anterior region of the midgut, although expression levels are less uniform throughout this domain. Non-visceral mesoderm cells also stain (mostly out of the plane of focus), which is observed in the wild-type as well. (E) *dpp303lacZ* expression in the wild-type. Note that although expression is weaker than observed with the other reporter constructs, it is still easily detected in PS7 (arrowhead). Weak expression of β -galactosidase is detected in the hindgut region (arrow). (F) *dpp303lacZ* expression in embryos lacking *exd* is nearly absent; only weak expression is observed in a few cells of PS7 (arrowhead). These embryos still express β -galactosidase in the hindgut region (arrow) and hence carry the transgene. (G) *dpp303lacZ* expression in heat-shocked wild-type embryos carrying the *HS:Ubx* transgene. Ectopic expression of β -galactosidase is detected anterior to PS7. (H) *dpp303lacZ* expression in heat-shocked embryos lacking *exd* and carrying *HS:Ubx*. β -Galactosidase is not expressed ectopically although these embryos carry the reporter construct, as expression in the hindgut region is observed (arrow).

constriction also fails to form. Clearly, loss of *exd* expression has profound effects not only on the expression of homeotic target genes, but also on morphogenesis.

Discussion

Mutations in *exd* cause homeotic transformations by altering the morphological consequences of homeotic selector gene activity (Peifer and Wieschaus, 1990). For example, when *exd* levels are reduced in the ectoderm, the homeotic selector gene *Ubx*, which normally specifies the identity of the first abdominal segment, specifies a first abdominal segment with third abdominal segment morphology. Genetic studies have demonstrated that *exd*

does not regulate the expression of the homeotic selector genes, nor is *exd* a downstream gene, as its function is not dependent upon homeotic regulation (Peifer and Wieschaus, 1990). Rather, *exd* acts in parallel to the homeotic selector genes. Nonetheless, from these genetic and morphological studies the mechanism of how *exd* affects a homeotic selector gene's ability to specify correct segmental identities remained elusive. Part of the answer lies in the fact that EXD possesses a homeodomain (Rauskolb *et al.*, 1993) and, hence, presumably participates in segmental identity specification by regulating gene expression. This fact suggested two possible models for *exd* function. One possibility was that EXD regulated a set of downstream genes distinct from homeotic targets.

Fig. 5. Embryonic midgut morphogenesis is aberrant in *exd* mutants. To visualize the gastric caeca and the constrictions of the midgut, embryos were stained with antibodies directed against muscle myosin. A comparison of the midgut in (A) wild-type and (B) *exd* embryos at stage 13 and in (C) wild-type and (D) *exd* embryos at stage 14, when the gut assumes an ellipsoid shape, shows that they have similar morphologies. (E) The midgut of a stage 16 wild-type embryo is subdivided into compartments because of the formation of three constrictions (arrows). The gastric caeca have also evaginated from the anterior end of the midgut (arrowhead). (F) In stage 16 *exd* mutant embryos the midgut is not subdivided into compartments because the constrictions fail to form. These embryos also do not have gastric caeca.

In this view, the final segment identity would result from the combined expression of both *exd* and homeotic selector target genes. Alternatively, the link between the homeotic selectors and EXD could be greater, in that they would control the expression of the same targets. The data presented in this paper demonstrate that EXD and the homeotic selector proteins coordinately regulate at least some of the same downstream genes, and that EXD is, in fact, an indispensable component in this regulation.

The expression patterns of the examined homeotic target genes, *dpp*, *wg* and *tsh*, are all altered in the visceral mesoderm of embryos lacking *exd* product, while the expression of their respective regulators, *Ubx*, *abd-A* and *Antp*, remains normal. Thus, the expression of these three downstream genes is not only dependent upon the activity of the selector proteins themselves, but also upon *exd*. We have further shown that both *exd* and *Ubx* can positively co-regulate *dpp* expression via the same 303 bp minimal enhancer. Importantly, *exd* mutations affect the activity, not the expression, of UBX. Even when UBX is expressed in *exd* mutant embryos ubiquitously under a heterologous promoter, it is still incapable of activating *dpp* enhancer expression. Our results thus clearly demonstrate that in *exd* mutant embryos the homeotic selector proteins are, in many instances, incapable of appropriately regulating their downstream target genes.

One potential mechanism for how EXD affects the ability of the selector proteins to regulate these targets is that EXD could interact directly with the selector proteins and enhance their specificity for target DNA sequences. Studies using other homeodomain proteins, such as $\alpha 2$ and $\alpha 1$ (Goutte and Johnson, 1993), UNC-86 and MEC-3 (Xue *et al.*, 1993) or the Paired class of homeodomain

proteins (Wilson *et al.*, 1993), have demonstrated that these proteins bind cooperatively as homo- or heterodimers to DNA, thereby increasing binding affinity. By analogy, EXD and the selector proteins may form stable heterodimers, which serve to increase the affinity of the selector proteins for specific DNA sites. Consequently, this interaction may allow for the occupancy of low affinity sites within a promoter by a selector protein, thereby enabling the regulation of genes that would normally not be regulated in the absence of *exd*. Consistent with this model is the finding that the 303 bp *dpp* enhancer contains both high and low affinity UBX binding sites (Capovilla *et al.*, 1994) and our observation that in the absence of *exd*, *Ubx* is incapable of promoting *dpp303lacZ* expression. Thus, we can imagine that in the absence of *exd*, only the high affinity sites are bound by UBX, which would be insufficient for transcriptional activation. In contrast, in the presence of *exd*, both high and low affinity sites would be bound by UBX, resulting in *dpp303lacZ* expression. Interestingly, *in vitro* studies have demonstrated that the affinity of UBX for the *dpp* enhancer is elevated in the presence of EXD (S.-K.Chan and R.Mann, personal communication). In a similar manner, EXD could be required for efficient binding of ANTP and ABD-A to their respective targets, *tsh* and *wg*.

Although the homeotic selector proteins and *exd* are both required for the regulation of some target genes, each can function independently as well. In all tissues in which they are expressed, the homeotic selector proteins regulate each other's expression, although the exact nature of this cross-regulation varies depending on the tissue examined. Given that in *exd* embryos *Ubx*, *Antp* and *abd-A* are expressed in a normal spatial and temporal pattern, they

must retain the ability to cross-regulate even in the absence of any *exd* product. We have also demonstrated that regulation by *abd-A* of its downstream target gene *dpp* can function independently of *exd*. Normally, *abd-A* represses *dpp* expression in the midgut posterior to PS7, both in the wild-type and in embryos where ectopic expression of *Ubx* induces a broad band of *dpp* in the anterior midgut (Reuter *et al.*, 1990). We have shown here that *dpp* is not expressed posterior to PS7 in embryos lacking *exd*, even when ectopic *Ubx* expression has been induced. This result indicates that *abd-A* does not require *exd* to repress *dpp* and contrasts with the dependence of *abd-A* on *exd* in the positive regulation of *wg*.

In addition, *exd* itself can function independently of the known homeotic selector proteins in its repression of *dpp* in parasegments anterior to PS7. The ectopic *dpp* expression in these parasegments in the absence of *exd* is not mediated by the homeotic gene that is known to be required for *dpp* expression, *Ubx*, as *exd Ubx* mutant embryos retain ectopic *dpp* expression. Nor is the anterior *dpp* expression regulated by the homeotic genes normally expressed in these parasegments. *Scr* is not expressed in the visceral mesoderm of embryos lacking *exd* product and *exd Antp* mutants still ectopically express *dpp*. Clearly, there is an as yet unidentified protein in the visceral mesoderm which in the absence of *exd* allows for tissue-specific expression of *dpp* in the anterior midgut. By using reporter constructs, the sites within the *dpp* regulatory region that mediate this ectopic expression have been localized to the 371 bp of the *dpp* regulatory region present in *dpp674lacZ*, but not in *dpp303lacZ*.

In conclusion, the identification of several genes as targets of both EXD and the homeotic selector proteins now allows us to determine how EXD and the homeotic selector proteins act in concert to regulate target gene expression. Subsequent studies will also be needed to explain such complexities as the difference between the dependence of ABD-A on EXD in the positive regulation of *wg* and the EXD-independence of ABD-A in the negative regulation of *dpp*. Since much of the regulatory network in the specification of segmental identities has been conserved, we suggest that co-regulation of target genes by Pbx and the Hox proteins will also occur. Coordinate regulation of downstream genes by the EXD/Pbx and HOM/Hox proteins, and the potential direct interaction between them, may present a means by which they achieve the correct pattern and levels of expression of downstream genes involved in the specification of different cellular fates during animal development.

Materials and methods

Fly stocks and production of germline clones

exd alleles used were *XP11* and *YO12*, both of which come from the zygotic screen of Wieschaus *et al.* (1984). The stocks and procedure used to generate female germline mosaics are described in Rauskolb *et al.* (1993). A line carrying *exd^{YO12}* and the *FRT* at cytological location 18D was also made. Female germline mosaics for either *exd^{XP11}* or *exd^{YO12}* were crossed to *ORE-R* males. In all figures wild-type refers to the progeny derived from these females fertilized with an X chromosome from these males. The expression patterns do not differ from the wild-type. The sibling embryos are mutant for *exd* because they did not receive a wild-type copy of *exd*; these *exd* mutant embryos were identified by their segmental fusions (Peifer and Wieschaus, 1990). To generate embryos that were *exd Antp* or *exd Ubx* from female *exd*

germline mosaics, the following stock and crosses were used: *exd^{XP11} FRT 18D/FM7*; *Antp^{W10}/TM2 Ubx¹³⁰* females were crossed to *ovo^{D2} FRT 18D/Y*; *FLP³⁸/FLP³⁸* males. After heat-shock treatment and eclosion, virgin females of the genotype *exd^{XP11} FRT 18D/ovo^{D2} FRT 18D*; *+FLP³⁸*; *+Antp^{W10}* or *TM2 Ubx¹³⁰* were crossed to *Antp^{W10}* or *Df(3R)P2* (i.e. *Ubx abd-A*) males respectively. Only one-eighth of the progeny completely lack *exd* and *Antp* or *Ubx*. These embryos were identified by segmental fusions indicative of the loss of *exd* and by the absence of staining with antibodies directed against *Antp* or *Ubx* respectively. The stock carrying *HS:Ubx* is described by Mann and Hogness (1990). The fly line carrying the *dpplacZ* reporter construct *dpp4000lacZ* was made with a 4 kb *Sal*RI fragment corresponding to coordinates ~72.5–76.5 in the *dpp* genomic map (St Johnston *et al.*, 1990), while the lines *dpp674lacZ* and *dpp303lacZ* are derived from subfragments from this genomic region. A description of these lines can be found in Capovilla *et al.* (1994). The mutants *Antp^{W10}* and *Df(3R)P2* and all balancers are described in Lindsley and Zimm (1992).

Heat-shock treatments

To induce ubiquitous expression of *Ubx* in embryos lacking *exd*, female *exd* germline mosaics were crossed to males carrying the *HS:Ubx* transgene. Embryos were collected for 12 h and then heat-shocked three times for 30 min at 37°C, with a recovery period of 30 min at 18°C between heat-shocks. The embryos were then placed at room temperature and after 2.5–3 h fixed and processed to determine the expression pattern of *dpp*. To determine the effects of ubiquitous *Ubx* expression on *dpp303lacZ* expression, female *exd* germline mosaics were crossed to the male progeny of flies with the *HS:Ubx* transgene crossed to flies with the *dpp303lacZ* transgene. Heat-shock treatments were done as described. Embryos were then stained for β -galactosidase expression. Half of the wild-type embryos that have received *dpp303lacZ* will also ubiquitously express *Ubx*; ectopic expression of *dpp303lacZ* was observed in 41/79 wild-type embryos, of which the embryo in Figure 4G is representative. The embryo in Figure 4H is representative of 47/50 embryos that were mutant for *exd* and carried *dpp303lacZ*. Half (i.e. 25) of the embryos should have also received the *HS:Ubx* transgene and thus, despite not being able to determine whether this particular embryo received the *HS:Ubx* transgene, we still conclude that *HS:Ubx* has no effect on the expression of *dpp303lacZ* in *exd* mutant embryos. The other three embryos had significant levels of β -galactosidase expression in about five cells of PS7 at stages 13–14, but ectopic expression of *dpp303lacZ* was never observed.

Histochemical stains

Whole-mount *in situ* hybridizations were carried out as described in Rauskolb *et al.* (1993). The following probes were used: the *dpp* cDNA was obtained as a 3.5 kb *Hind*III–*Eco*RI fragment from pBEh1 (St Johnston *et al.*, 1990), the *tsh* cDNA was obtained as a 2.5 kb *Eco*RI fragment (Fasano *et al.*, 1991) and the *wg* cDNA was not isolated from pSP65 (Rijsewijk *et al.*, 1987), but rather the whole plasmid was labelled. Immunohistochemical stains were done according to the protocol described in Irvine *et al.* (1991), except that PBS + 0.2% BSA was used instead of BSS. The Vector SG Substrate Kit For Peroxidase (Vector Laboratories, Inc.) was used for staining detection. The antibodies used were the following monoclonal antibodies: FP3.38 recognizing *Ubx* (White and Wilcox, 1984), 8C11 recognizing *Antp* (Condie *et al.*, 1991), mAbDMabdA.1 recognizing *abd-A* (Kellerman *et al.*, 1990) and 6H4.1 recognizing *Scr* (Glicksman and Brower, 1988). Polyclonal antibodies directed against *Drosophila* muscle myosin (Kiehart and Feghali, 1986) and β -galactosidase (Cappel) were also used. Antibody staining and *in situ* hybridization double labelling was done essentially as described in Manoukian and Krause (1992), but the embryos were fixed as in the immunohistochemical stains described above and 1.5 mM DTT was added to the PBTH solution to maintain RNasin (Promega) activity. Embryos were staged according to Campos-Ortega and Hartenstein (1985).

Acknowledgements

We thank Matthew Scott and Laura Mathies, Richard Mann and Siu-Kwong Chan and Juan Botas for communicating unpublished results. We thank Gary Mansfield and Dan Kiehart, Dianne Mattson and Ian Duncan and Dan Brower for antibodies, Rob Ray for the *dpp* cDNA, Stephen Kerridge for the *tsh* cDNA, Richard Mann for the *HS:Ubx* flies and Juan Botas for *dpplacZ* flies. We appreciate the critical and thoughtful

reading of this manuscript by Ken Irvine, Rob Ray and Eyal Schejter. This research was funded by National Institutes of Health grant 5R01HD22780 to E.W.

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Received on May 3, 1994; revised on May 20, 1994

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