Synergistic activation of a *Drosophila* enhancer by HOM/EXD and DPP signaling

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The homeotic proteins encoded by the genes of the *Drosophila* HOM and the vertebrate HOX complexes do not bind divergent DNA sequences with a high selectivity. *In vitro*, HOM (HOX) specificity can be increased by the formation of heterodimers with Extradenticle (EXD) or PBX homeodomain proteins. We have identified a single essential Labial (LAB)/EXD-binding site in a Decapentaplegic (DPP)-responsive enhancer of the homeotic gene *lab* which drives expression in the developing midgut. We show that LAB and EXD bind cooperatively to the site *in vitro*, and that the expression of the enhancer *in vivo* requires *exd* and *lab* function. In addition, point mutations in either the EXD or the LAB subsite compromise enhancer function, strongly suggesting that EXD and LAB bind to this site *in vivo*. Interestingly, we found that the activity of the enhancer is only stimulated by DPP signaling significantly upon binding of LAB and EXD. Thus, the enhancer appears to integrate positional information via the homeotic gene *lab*, and spatiotemporal information via DPP signaling; only when these inputs act in concert in an endodermal cell is the enhancer fully active. Our results illustrate how a tissue-specific response to DPP can be generated through synergistic effects on an enhancer carrying both DPP- and HOX-responsive sequences.

Keywords: DPP signaling/endoderm induction/
extradenticle/homeotic genes

Introduction

During the development of multicellular organisms, cells have to be able to sense their position with respect to the major body axis, and maintain this information during subsequent cell divisions up to the adult stages. A group of genes which play a major role in conferring and maintaining positional information is located in the homeotic selector gene complex (HOM-C) in *Drosophila* and the homologous HOX gene complexes in vertebrates (Lewis, 1978; Hayashi and Scott, 1990; McGinnis and Krumlauf, 1992; Gehring *et al.*, 1996). All of the homeotic genes in these complexes encode homeodomain proteins and are thought to fulfill their function by regulating

distinct sets of downstream target genes (Botas, 1993; Mann, 1995; Gehring *et al.*, 1996; Mann and Chan, 1996; Graba *et al.*, 1997). However, the molecular mechanisms by which the different homeotic genes control the formation of specific structures in given places are far from being understood.

The identification of *in vivo* HOM/HOX target sites has provided insight into the molecular mechanisms governing the selective regulation of genes by homeotic proteins. Guided by observations made in genetic studies which identified the *extradenticle* (*exd*) gene as an important regulator of homeotic gene function (Peifer and Wieschaus, 1990), biochemical analyses have shown that several homeotic proteins bind to *in vivo* target sites cooperatively with the homeodomain protein EXD. This cooperative recognition of sequences in target enhancers contributes both to a higher affinity of the heteromeric complex (when compared with the monomeric proteins) and to a more selective recognition of target elements (Mann, 1995; Mann and Chan, 1996).

One of the best characterized HOM/EXD-binding sites is present in a 20 bp oligonucleotide, repeat 3, which was identified in the 5' autoregulatory region of the mouse *Hoxb-1* gene (Pöpperl *et al.*, 1995). Labial (LAB) protein and its mammalian ortholog HOXB-1 are both able to bind cooperatively with EXD to this site, whereas other HOM/HOX proteins, such as Ultrabithorax (UBX) or HOXB-4, cannot (Pöpperl *et al.*, 1995; Chan and Mann, 1996; Chan *et al.*, 1996). Furthermore, when *lacZ* reporter constructs containing three tandem copies of repeat 3 were introduced into either *Drosophila* or mouse embryos, *lacZ* expression patterns resembling the endogenous *lab* and *Hoxb-1* patterns, respectively, were generated (Chan *et al.*, 1996). However, no *Drosophila* cis-regulatory element has been reported to contain a functional LAB/EXD site resembling the original repeat 3 sequence. The identification of such *in vivo* binding sites would allow the study of the interaction of homeotic proteins with co-factors and other transcriptional regulators in their natural environment, using genetic and biochemical methods.

In addition to the cell-autonomous maintenance of positional information, cells have to communicate to allow a concerted development of all tissues and organs. Members of the transforming growth factor- β (TGF- β) superfamily of signaling molecules play important roles in such intercellular communication processes (Massagué, 1996). One of the *Drosophila* members of this superfamily, Decapentaplegic (DPP; Gelbart, 1989), has been shown to be both upstream and downstream of the homeotic genes; on the one hand, DPP regulates the expression of certain homeotic genes, on the other hand, *dpp* transcription is regulated in some tissues by homeotic gene products (Bienz, 1994, 1996).

Here, we report the identification of a single high

affinity LAB/EXD-binding site in an enhancer of the *lab* gene which drives expression in endodermal cells in response to DPP signaling (Tremml, 1990; Tremml and Bienz, 1992; Eresh *et al.*, 1997). We show that point mutations in either the LAB or the EXD subsite abolish cooperative DNA binding *in vitro*, and strongly reduce the activity of the enhancer *in vivo*. In addition, both *lab* and *exd* are genetically required for high level expression, strongly suggesting that LAB and EXD bind to these sites *in vivo*. Furthermore, we observe that the DPP responsiveness of the enhancer (Eresh *et al.*, 1997) is largely abolished in the absence of EXD and LAB. Our results indicate that the activity of this *lab* enhancer requires a combined and synergistic input from the DPP signaling cascade on the one hand, and from the HOM selector gene product LAB on the other hand. Our results illustrate how a tissue-specific response to DPP can be generated through synergistic effects on an enhancer carrying both DPP- and HOM-responsive sequences.

Results

A 48 bp element is essential for the activity of a DPP-dependent enhancer of the *lab* gene

It has been reported previously that a 550 bp fragment located 2.5 kb upstream of the *Drosophila lab* gene (HZ550; hereafter referred to as lab550 or 1/546) is activated in the endodermal cell layer in response to the DPP signaling molecule secreted by visceral mesoderm parasegment 7 cells (Tremml, 1990; Tremml and Bienz, 1992; Eresh *et al.*, 1997). In an attempt to define a minimal DPP-responsive element, we made terminal deletions and examined the expression patterns driven by the shortened elements in transgenic embryos (constructs are shown in Figure 1 and expression in transgenic embryos in Figure 2). As a result of this analysis, we identified a 45 bp fragment (located between nucleotides 48 and 92 of the lab550 element) which was essential to drive high levels of expression in the endoderm. While the deletion of the first 48 bp of lab550 had no effect on its expression (Figure 2A and B), the removal of an additional 45 bp resulted in an almost complete loss of expression of the reporter gene (Figure 2C, compare 48/546 with 92/546). Two very short constructs each containing the essential 45 bp (1/95 and 48/95) were also expressed in the midgut endoderm, although at a lower level (and slightly more posterior) than the full-length element (Figure 2D and F; see also Figure 6). The removal of only 9 bp from the 3' end of element 1/95 resulted in a very strong reduction in expression (construct 1/86; Figure 2E). Based on the results obtained with these deletion constructs, we concluded that a *cis*-acting element essential for expression in the central midgut endoderm must be located within this 45 bp region.

Expression of the *lab* enhancer requires *lab* and *exd* function

Sequence inspection of the essential 45 bp region suggested that it could be involved in conferring homeotic and signaling-dependent regulation to lab550. We identified a sequence element (TGATGGATTG; shown in bold and italics on the bottom strand in Figure 1) which is identical in nine out of ten positions to a sequence element of the

Fig. 1. Transgenic constructs and mutations in a LAB/EXD-binding sequence. (A) Wild-type and deletion constructs were named according to the extent of sequences present. The wild-type enhancer is 546 bp long and corresponds to HZ550 in Tremml and Bienz (1990) (see Materials and methods). We refer to this construct as lab550 or 1/546. The level of expression in the central midgut endoderm has been determined semi-quantitatively in several lines for each construct; strongest expression was seen with the wild-type construct, whereas 1/95 and 48/95 were significantly weaker but clearly detectable in the midgut. The sequence of an essential 48 bp region is shown below the constructs. A 10 bp sequence corresponding to a putative LAB/EXD-binding site is displayed in bold. The exact endpoints of deletions 1/86 and 92/546 are indicated with open arrows. (B) Point mutations introduced into the LAB/EXD site are shown, along with the nomenclature of the corresponding transgenic constructs. Mutated residues are printed in bold and further indicated with an asterisk.

mouse *Hoxb-1* upstream regulatory region (TGATGG-ATGG; Pöpperl *et al.*, 1995) that previously has been shown to be capable (in an oligomerized form) of conferring LAB/EXD-dependent expression in the *Drosophila* embryo, both in the midgut and in the head (Chan *et al.*, 1996, 1997). This element might indeed be involved in the activation of lab550, since the 9 bp deletion from construct 1/95 to 1/86 (which abolishes expression of the element) removes part of the putative LAB/EXD site (Figures 1 and 2). The recent finding that the nuclear localization of the EXD homeodomain protein in the developing endoderm is regulated by DPP (Mann and Abu-Shaar, 1996) suggests that the LAB/EXD-binding site might mediate part of the DPP responsiveness of the lab550 enhancer.

To investigate whether lab550 as well as the short 48 bp enhancer (48/95) were indeed *lab* dependent, we analyzed

Fig. 2. Expression of transgenic constructs in stage 14 embryos. β -Gal expression driven by the different constructs. Strong expression in all cells of the expression domain is seen for constructs 1/546 (A) and 48/546 (B). Only a few cells (on average 1–4 cells per embryo) are detected in embryos transgenic for construct 92/546 (C). Expression in the developing midgut is clearly detected with the short elements 1/95 (D) and 48/95 (F), but expression levels are lower than with constructs 1/546 and 48/546, and endodermal cells within the expression domain are not stained uniformly. Construct 1/86 (E) does not drive expression in the midgut. All constructs are also expressed in cells of the amnioserosa and in lateral epidermal cells which served as a control for the presence of the construct in the stained embryos.

the expression pattern driven by these elements in a *lab* null mutant background (Diederich *et al.*, 1989). Expression of *lab550* was strongly reduced in stage 14 embryos in the absence of LAB, and remained detectable at low levels in 2–4 cells only (Figure 3B; compare to Figure 3A). Expression driven by the 48 bp element was not detectable at all in the midgut of *lab* mutant embryos, demonstrating an absolute requirement for *lab* function in the activation of 48/95 (Figure 3C). In contrast, the residual expression of the deletion construct 92/546 (1–4 cells, occasionally, in the central midgut endoderm; Figure 2C) was not affected by the absence of *lab* (data not shown). This finding is in line with the absence of the putative LAB/EXD-binding site in construct 92/546.

We have also analyzed the expression pattern of the full-length construct in the absence of the *exd* gene product. To test the requirement for *exd*, which is a highly expressed maternal gene (Rauskolb *et al.*, 1993), we generated females with mosaic *exd* mutant germlines. When crossed to males carrying the *lab550* element, virtually no expression was observed in embryos derived from *exd* mutant germline clones (Figure 3D). These experiments demonstrate that the expression of the DPP-dependent *lab550* enhancer critically depends on the presence of the two homeodomain-containing transcriptional regulators EXD and LAB.

Fig. 3. LAB and EXD protein are required for high levels of expression of the *lab* enhancer. To identify *lab* mutants, embryos shown in (A), (B) and (C) were stained in brown for β -gal and in blue for the endogenous LAB protein. (A) Wild-type embryo transgenic for construct 1/546. (B) Expression of construct 1/546 in an embryo lacking LAB protein; only weak expression in a few cells is detectable. In stage 16 *lab* mutant embryos, expression levels are somewhat increased (see also Tremml and Bienz, 1992); apparently, DPP-inducible enhancer activity in late embryos is less dependent on *lab* (data not shown). (C) The short element 48/95 is not expressed at detectable levels in the endoderm in *lab* mutants. (D) Only weak and scattered expression of the *lab550* enhancer (1/546) is detected in embryos derived from females carrying *exd* mutant germline clones. (E) Expression pattern of an embryo transgenic for a construct (5CRE) carrying oligomerized CRE-binding sites; note the expression in the anterior and the central midgut. (F) Expression of the 5CRE construct is also evident in embryos lacking EXD protein; the expression domain is altered but follows the pattern of *dpp* expression in the visceral mesoderm of *exd* mutants (data not shown; see Rauskolb *et al.*, 1994).

Activity of the *lab550* enhancer requires the LAB and the EXD half-sites

To find out whether the genetic requirement for *lab* and *exd* was linked to the single putative LAB/EXD site in *lab550*, we tested whether the introduction of point mutations in the EXD or LAB subsites in the context of the full-length *lab550* enhancer would impair expression of the latter. The strategy to inactivate either the EXD or the LAB half-site, exclusively, was based on previous biochemical analysis (Chan and Mann, 1996; Chan *et al.*, 1996, 1997). The mutations we introduced are shown in Figure 1. Indeed, inactivation of either the LAB subsite by a single mutation (*lab*^{m1}, Figure 4B), or of the EXD subsite by the mutation of one (*exd*^{m1}; Figure 4C) or two adjacent bases (*exd*^{m2}; Figure 4D) resulted in a severe reduction in expression, mimicking the expression of the full-length (wild-type) element in *lab* or *exd* mutant backgrounds (compare with Figure 3B and D). Mutation of both the EXD and the LAB subsite resulted in an even stronger reduction of expression (*exd*^{m1}*lab*^{m1}; Figure 4E).

Fig. 4. Point mutations in the putative LAB/EXD-binding site strongly reduce expression of the *lab* enhancer. (A) Expression of the full-length enhancer (*lab550* or 1/546) is compared with the expression of constructs harboring one or two mutations in the context of the full-length element. Expression levels were severely reduced upon the introduction of a mutation in the LAB subsite (B, *lab*^{m1}) or the EXD subsite (C, *exd*^{m1}), or upon the introduction of two point mutations in the EXD site (D, *exd*^{m2}) or the LAB/EXD site (E, *exd*^{m1}*lab*^{m1}). Changing the two base pairs in the center of the LAB/EXD site from GG to TA somewhat reduced the expression level, but did not alter the domain of expression (F, *lab*^{m2}). Note that no expression is detectable in the head region with construct *lab*^{m2} (F).

To confirm that EXD and LAB indeed bind cooperatively to the LAB/EXD site which we identified in *lab550*, we performed band shift experiments with labeled oligonucleotide probes and purified proteins (Figure 5A). The TGATGGATTG wild-type site formed complexes with EXD and LAB, but not with EXD or LAB alone. The reduced affinity of the point mutants in the EXD or the LAB subsite for LAB–EXD complexes was confirmed by band shift experiments; both mutations abolish complex formation (Figure 5A). Taken together, these results strongly suggest that EXD and LAB are binding directly to the *lab* enhancer element *in vivo*, consistent with the genetic requirement for *exd* and *lab* function.

It has been reported previously that a two base pair change (GG→TA) in the minimal LAB/EXD-responsive element repeat 3 can switch the *in vivo* specificity from *lab* to *Deformed* (*Dfd*) (Chan *et al.*, 1997). To test whether we could similarly alter the expression pattern of the *lab550* enhancer, we introduced the same two base pair mutation in the LAB/EXD site of *lab550*, and analyzed the expression pattern in transgenic embryos. Midgut expression was clearly still detectable (although at somewhat reduced levels), and we never observed expression in the head region (*lab*^{m2}; Figure 4F). Thus, although DFD can bind to the TA site *in vitro* (see Figure 5), it is incapable of activating the TA-containing *lab550* enhancer in transgenic embryos in the DFD expression domain. This suggests that factors in addition to LAB and EXD

determine the germ layer specificity of the *lab550* enhancer element (see also Discussion). This is also evident from the observation that construct 92/546 (which lacks the LAB/EXD site) is still expressed in the endoderm (Figure 2C).

Strong induction of the *lab* enhancer by DPP requires LAB and EXD

In several previous reports, it has been shown that *lab550* represents a DPP-responsive enhancer (Tremml, 1990; Tremml and Bienz, 1992; Eresh *et al.*, 1997). We have shown above that in *lab* and *exd* mutant embryos, the *lab550* enhancer is not strongly expressed (Figure 3), although DPP is present in *lab* mutants and (albeit in an altered pattern) in the visceral mesoderm of *exd* germline clone-derived embryos (Rauskolb and Wieschaus, 1994). In contrast to *lab550*, other DPP-dependent enhancers (such as the cAMP response element, 5CRE; Eresh *et al.*, 1997) do respond to DPP in *lab* mutant embryos (data not shown) and in embryos lacking maternal and zygotic *exd*, confirming that DPP signaling *per se* is not impaired in the endoderm in the absence of *lab* or *exd* (Figure 2G and H; see also Discussion). Since the endogenous *lab* gene is strongly induced by DPP (Immerglück *et al.*, 1990; Reuter *et al.*, 1990; Grieder *et al.*, 1995), the DPP responsiveness of *lab550* expression could, in principle, be entirely due to an indirect autoregulatory feedback loop through the LAB/EXD site. However, more recent results suggest that CREB-binding sites act as targets for DPP signaling in *lab550* (Eresh *et al.*, 1997).

To assess the role of DPP signaling in the expression and regulation of various sub-elements of *lab550*, we have analyzed different enhancer constructs with respect to their DPP responsiveness. As reported previously (Tremml and Bienz, 1992; Eresh *et al.*, 1997), *lab550* is not expressed in *dpp*^{s4} mutants which lack *dpp* expression in the visceral mesoderm (Figure 6B). In *abdA* mutants, in which *dpp* is expressed in the entire posterior midgut, *lab550* is expanded posteriorly (Immerglück *et al.*, 1990) (Figure 6C). In addition, *lab550* can be induced to high levels in most endodermal cells upon ubiquitous DPP expression (Figure 6D). Thus, the *lab550* enhancer not only strictly requires DPP for expression, but responds strongly to DPP upon genetic manipulation of the expression pattern of *dpp*. In all of the experiments shown in Figure 6, the expression of the *lab550* enhancer closely follows the expression of the endogenous *lab* gene.

We next analyzed the residual expression of the deletion construct 92/546 in the same genetic backgrounds. Because the expression of this construct does not depend on *lab* function, its activity is not influenced by *dpp* indirectly due to the induction of the endogenous *lab* gene. In *dpp*^{s4} mutants, expression driven by the 92/546 construct is abolished, demonstrating that the residual, *lab*-independent expression depends on DPP (Figure 6F). Consistent with this observation, low levels of expression are seen in the entire posterior midgut in *abdA* mutants (Figure 6G); upon ectopic expression of DPP, scattered cells expressing the 92/546 construct were found in the posterior midgut and (rarely) in the anterior midgut (Figure 6H). This demonstrates that the sequences 3' to the LAB/EXD site behave, by themselves, as a weak DPP response element. Similar conclusions were reached based on the expression

Fig. 5. Cooperative binding of LAB and EXD requires both the LAB and EXD half-sites. ³²P-Labeled oligonucleotides containing the wild-type (WT), exd^{m1}, exd^{m2}, lab^{m1} or lab^{m2} LAB/EXD-binding sites were mixed with EXD and LAB (**A**) or EXD and DFD (**B**) proteins as indicated, and complexes were resolved on native polyacrylamide gels. LAB-EXD complex formation is reduced by a single base pair change (exd^{m1}) and abolished by a two base pair change (exd^{m2}) in the EXD half-site. LAB-EXD complex formation is abolished by a single base pair change in the LAB half-site (lab^{m1}) but only weakly reduced by the GG→TA change in the LAB half-site (lab^{m2}). As was previously observed (Chan *et al.*, 1997), DFD/EXD prefers to bind the sequence 5'TGATTAAT rather than 5'TGATGGAT.

of a lab550 subfragment extending from approximately position 95 to position 300 (Tremml and Bienz, 1992).

We have also investigated the behavior of construct lab^{m1}exd^{m1} in the same genetic backgrounds. This construct behaved in a virtually identical manner to 92/546 (data not shown), a finding which is consistent with the requirement for LAB and EXD for strong DPP inducibility.

The low expression levels and the weak DPP inducibility of construct 92/546 (and exd^{m1}lab^{m1}) suggested that the expression of the lab550 element might be driven mainly by the 48 bp element which contains the LAB/EXD site, and that the adjacent sequences play only a moderate role in the control of the expression domain and/or expression level. To test this possibility, we have analyzed the expression domain of 48/95, and tested its response to DPP. Although the expression of the short element in the central midgut strictly depends on *lab* function (Figure 3C), its expression domain is shifted posteriorly with respect to lab550 and to the expression domain of the endogenous LAB protein (Figure 6M and legend). Surprisingly, the 48 bp element did not respond to DPP very efficiently; only weak activation was observed in the posterior midgut upon ubiquitous expression of DPP, and no DPP-dependent induction was observed in anterior endodermal cells (Figure 6P). More surprisingly, the expression of 48/95 was lost in *abdA* mutants, demonstrating that the element requires a signaling input from the visceral mesoderm in addition to DPP; this additional input is presumably lost in *abdA* mutants. Similar observations were made when the 1/95 element was analyzed in the same genetic backgrounds (Figure 6I–L). These results contrast with those obtained with lab550, which is strongly responsive to DPP in most cells of the midgut (Figure 6C and D), and demonstrate that although the 48 bp element

containing the LAB/EXD site is essential for expression of lab550, it is not sufficient to reproduce the properties of the latter.

Our experiments demonstrate that by themselves, neither the 5' sub-elements (1/95 and 48/95) nor the 3' sub-element (92/546) of lab550 can recapitulate the behavior of the latter, either with respect to the expression domain and the expression levels, or with respect to the DPP inducibility. Therefore, it appears that input through both sub-elements synergizes to allow for a response to homeotic gene products and to DPP signaling (see Discussion).

Discussion

A lab enhancer represents a direct autoregulatory target

We have identified a single binding site for a LAB/EXD heterodimer in an autoregulatory element of the *lab* gene (lab550). Based on the following observations, we suggest that this site represents an *in vivo* target for the homeotic protein LAB and its co-factor EXD: (i) genetically, the expression of lab550 depends strongly on the presence of LAB and EXD (Figure 3); (ii) an essential 48 bp region of the lab550 element contains a site to which LAB/EXD bind cooperatively *in vitro*, and which is identical at nine out of 10 positions to a HOXB-1/PBX site identified in the 5' promoter region of the mouse *Hoxb-1* gene (Pöpperl *et al.*, 1995); (iii) point mutations in the LAB and EXD subsites abolished cooperative DNA binding *in vitro* (Figure 5) and resulted in strong reduction of the full-length lab550 enhancer activity *in vivo* (Figure 4). Taken together, the results of the genetic, the reverse genetic and the biochemical analyses support the view that lab550 is

Fig. 6. Expression of the wild-type *lab550* enhancer is, in contrast to isolated sub-elements, strongly DPP responsive. Expression of the wild-type *lab550* enhancer (1/546) (A–D, horizontal lines), and constructs 95/546 (E–H), 1/95 (I–L) and 48/95 (M–P) were analyzed in wild-type embryos (A, E, I and M; vertical lines), *dpp^{ts4}* mutant embryos (B, F, J and N), *abdA* mutants (C, G, K and O) and upon ectopic expression of DPP (D, H, L and P). Note that both small constructs (I and M) are expressed in cells posterior to the second midgut constriction (arrow); these cells do not express the endogenous *lab* gene during these stages. The expression of all constructs strictly depends on DPP since none of them is active in *dpp^{ts4}* mutant embryos which lack DPP in the visceral mesoderm (B, F, J and N). The *lab550* enhancer is strongly expressed in most endodermal cells posterior to the first midgut constriction in *abdA* mutant embryos; these endodermal cells are adjacent to DPP-expressing visceral mesodermal cells (Immerglück *et al.*, 1990; Reuter *et al.*, 1990). The deletion construct 95/546 which lacks the LAB/EXD-binding site is also expressed in posterior endodermal cells (G), although it is weaker and more patchy than the *lab550* element. However, the short elements 1/95 (K) and 48/95 (O) are not expressed in *abdA* mutants and therefore do not respond to DPP in this mutant background. Element 1/95 drives weak expression in the most posterior endodermal cells in stage 15 embryos. *lab550* is strongly induced by ectopic DPP in most endodermal cells (D). The sub-elements 92/546 (H), 1/95 (L) and 48/95 (P) respond weakly to DPP in the posterior midgut, but cannot be induced by DPP in endodermal cells anterior to the normal expression domain (see H and L).

a direct *in vivo* target for the LAB/EXD complex. Lab550 represents the first *bona fide* *Drosophila* target for the LAB/EXD protein-protein complex, which has served as a paradigm to study how HOM/HOX genes regulate their targets. In contrast to most other HOM/HOX targets previously characterized, the *lab* enhancer represents a very simple situation in being regulated by the LAB/EXD complex through one single, essential site. The identification of an *in vivo* target site for a LAB/EXD complex in *Drosophila* allowed us to dissect the function of a HOM gene product on a natural target and its interaction with factors bound to neighboring sequences. These studies indicate that the LAB/EXD complex functions synergistically with other transcriptional regulators interacting with distant sequences in the lab550 element (see below).

It has been reported previously that the lab550 enhancer is only weakly *lab* dependent (Tremml and Bienz, 1992). One argument for this interpretation was the fact that lab550 is expressed in a domain in the central midgut endoderm which includes cells immediately preceding the

second midgut constriction, i.e. cells that do not induce endogenous *lab*. However, these cells contain LAB protein due to its earlier accumulation in the posterior midgut primordia (Mlodzik *et al.*, 1988; Diederich *et al.*, 1989). From the strongly reduced expression of *lab550* and from the complete lack of expression of the shorter 48/95 construct in *lab* mutants (in all the cells where these constructs are normally expressed, including the most posterior ones), we conclude that the low levels of LAB protein in the midgut primordia are crucial (and possibly sufficient) for the activation of the enhancer during midgut fusion. Indeed, it has been shown that the low levels of LAB in the endoderm primordia can activate promoters when they are fused to oligomerized LAB/EXD sites (Chan *et al.*, 1996).

EXD and LAB are necessary but not sufficient to activate the autoregulatory enhancer

Although EXD and LAB are required for the expression of *lab550*, they are not sufficient for its proper regulation. We found that the expression of a deletion construct

(1/227) which contains the LAB/EXD site plus flanking sequences is hardly detectable in the endoderm; a deletion construct which contains more 3' flanking sequences (1/311) is expressed at high levels (data not shown). More strikingly, the mutation of four CRE sites (all located 3' of the LAB/EXD site) results in reduced activity of the lab550 enhancer in the presence of the LAB/EXD-binding sequence (Eresh *et al.*, 1997). Therefore, although EXD and LAB bind to the element cooperatively and are crucially required for its activation, they are not sufficient to activate the nearby promoter in the absence of flanking sequences.

This observation is somewhat puzzling since we found that the small 48 bp element (48/95) drives *lab*-dependent expression in the central midgut, albeit at reduced levels (Figure 2). However, closer inspection of the expression pattern of construct 48/95 revealed that its expression domain extends more towards the posterior than the expression domain of the full-length construct (see Figure 6), and resembles the expression pattern of the oligomerized repeat 3 sequences in germ band-retracted embryos (Chan *et al.*, 1996; and data not shown); however, in contrast to repeat 3, 48/95 is not activated before the two endoderm primordia fuse at the end of germ band retraction, and does not drive expression in the head. Strikingly, we observed that the short 48/95 enhancer is only very moderately responsive to DPP, and does not respond to DPP at all in *abdA* mutant embryos (Figure 6O); this is in sharp contrast to the lab550 enhancer which is strongly DPP responsive. Therefore, although LAB/EXD drives expression through the short 48/95 element, this element is not capable of reproducing the original expression pattern; other sequences must restrict the expression domain, and confer the strong DPP inducibility to the lab550 enhancer.

Multiple inputs control the DPP responsiveness of the *lab* enhancer

How does DPP signaling affect the expression of the lab550 enhancer? The enhancer has been reported as a DPP response element based on the fact that all of its activity in the midgut requires *dpp* function, but very little of it is due to *lab* function; in addition, it drives expression one or two cells more posterior than the endogenous *lab* gene, thus apparently displaying *lab*-independent expression (Tremml and Bienz, 1992). Although these conclusions somewhat contrast our own, further analysis suggested that several CREs in the enhancer mediate the DPP responsiveness, directly or indirectly (Eresh *et al.*, 1997). More recently, *in vitro* binding sites for *Drosophila* MAD, a member of the SMAD protein family which are thought to act as co-activators of transcription in the DPP signaling pathway (Raftery, 1995; Sekelsky, 1995), have also been identified in the *lab* enhancer (Kim *et al.*, 1997). In addition, we found numerous binding sites for SCHNURRI, a putative transcription factor which is required for endodermal cells to respond to DPP (Arora *et al.*, 1995; Grieder *et al.*, 1995; unpublished).

Despite the presence of all these putative target sequences of the DPP signaling pathway, we demonstrated that the expression of the *lab* enhancer strongly depends on the presence of a 48 bp region which harbors an EXD/LAB site; in *exd* and *lab* mutant embryos, expression of

the element is strongly reduced. Since it recently has been shown that the translocation of EXD from the cytoplasm to the nucleus in the endoderm is controlled by DPP and Wingless (WG) (Mann and Abu-Shaar, 1996), and since the endogenous *lab* gene is strongly induced by DPP, it is likely that part of the DPP regulation of the *lab* enhancer enters through the EXD/LAB site. This is consistent with our findings and with the previous observation that in the absence of *lab* function, *dpp*-mediated induction is hardly working or working with reduced efficiency (Tremml and Bienz, 1992).

What is the role of the additional DPP response elements in the *lab* enhancer? Clearly, the activity of the enhancer also depends on the presence of CRE sites; similarly to our observations concerning the LAB/EXD site, mutations in the four CRE sites result in weaker expression of the enhancer (Eresh *et al.*, 1997). This suggests that on the full-length enhancer, the additional (direct or indirect) DPP input via the CRE and other sites is required in concert with the input through the LAB/EXD site, and the absence of either input impairs expression of the enhancer.

Our analysis has shown that neither the 5' sub-elements (1/95 and 48/95) nor the 3' sub-element (92/546) are significantly DPP responsive in the anterior midgut, and only weakly DPP responsive in the posterior midgut; this is in sharp contrast to the wild-type element which is extremely sensitive to ectopic DPP in most endodermal cells. Therefore, the two elements appear to act synergistically with respect to their response to DPP; only upon the addition of the weak DPP response element (92/546) to the LAB/EXD-containing element (48/95) is a *cis*-acting element generated which is strongly DPP inducible (and LAB/EXD dependent). We propose that the strong DPP responsiveness of the enhancer is limited, through synergistic interactions, to those cells in which the LAB/EXD site is occupied. The function of this dual requirement might be to insure that the enhancer activates transcription exclusively in cells located in the central portion of the midgut endoderm where low levels of LAB in the tip of the two endoderm primordia and DPP secreted from the visceral mesoderm parasegment 7 coincide.

Homeotic gene products and the cellular response to DPP

It has been shown previously that the cellular (transcriptional) response to DPP in the visceral mesoderm is largely dependent on the parasegmental provenance, and thus on the expression of a specific homeotic gene, of a given cell (Immergluck *et al.*, 1990; Reuter *et al.*, 1990; Staehling-Hampton and Hoffmann, 1994; Bienz, 1994, 1996). In the developing wing, it has been demonstrated that DPP specifies the anterior or posterior pattern depending on the compartmental provenance, and hence the state of activity of *engrailed*, of the responding cell (Zecca *et al.*, 1995). We have provided evidence that the activity of a DPP response element (lab550) depends directly on the presence of a homeotic selector gene product, LAB, and its co-factor EXD. This strict requirement limits the efficient DPP signal-dependent activation of the enhancer to cells carrying precise HOM-derived information concerning their position in the embryo. Thus, the direct requirement for a homeotic protein for the DPP inducibility

of an enhancer is in part responsible for the particular response of a cell to the DPP signal. It will be interesting to see whether other DPP response elements also carry essential binding sites for homeotic or other selector gene products.

Materials and methods

Fly stocks and transformants

Transformant lines were generated by standard procedures. All *Drosophila* reporter genes were generated by standard cloning procedures, and inserts were cloned into a *KpnI* and *Bam*HI site of the nuclear LacZ-encoding P element vector pC β (a gift of Konrad Basler). Mutations and deletions of the original 550 bp *ClaI* lab fragment (Tremml, 1990; Tremml and Bienz, 1992) were generated using a PCR-based approach. For each reporter construct, the *lacZ* expression pattern was determined for several independent transformant lines; in each case, the large majority of transformants of a given construct showed identical expression patterns.

UAS-dpp and *hs-gal4* transformants were provided by K.Basler and D.Nellen (Ruberte *et al.*, 1995). For the analysis of expression in mutant backgrounds, the following alleles were used: *dpp^{sc}* (Immerglück *et al.*, 1990), *abdA^{ml}* (Casanova *et al.*, 1987), *lab^{vdI}* (Diederich *et al.*, 1989) and *exd^{Xp11}* (see Chan *et al.*, 1996).

Mutagenesis

The 550 bp *ClaI* fragment was cut out of bs1.2 (Tremml and Bienz, 1992) and subcloned into pBluescript, KS. The 550 bp fragment was cloned into the *KpnI* and *Bam*HI site of pC β by a PCR-based approach. Using the primers LabKpn (gcgggtaccatcgatttaccataaattac) and LabBam (cgcggtacctaaccataaaagctatttc), the fragment was PCR amplified, cut with the restriction enzymes *Asp*718 and *Bam*HI and subcloned into the *Asp*718 (*KpnI*) and the *Bam*HI site of pC β ; thereby, a *KpnI* site was introduced 5' of the *ClaI* site and the 3' *ClaI* site was replaced by *Bam*HI. All other constructs were made similarly using primers LabKpn and LD311Bam (gctggatccaagtgtatggg) (1/311), LabKpn and LD227Bam (cgcggtaccgctctgtgtctc) (1/227), LabKpn and LD95Bam (gctggatccaattgatggattg) (1/95), LabKpn and LD86Bam (gctggatccggattgcccg) (1/86), Kpn48 (cgcggtacacctttgtcttgagc) and LabBam (48/546), and Kpn48 and LD95Bam (48/95). The point mutation in *lab^{ml}* was introduced by a two-step PCR amplification: the 5' end was amplified using the primers LabKpn and lab85rev (gatggttgcggcg), and the 3' end using Lab85 (cgggcaaacatcaatttg) and LabBam; the amplified fragments were purified and the full-length fragment amplified using primers LabKpn and LabBam. All other mutant constructs were generated accordingly.

Antibody stainings

The anti- β -Gal antibody was produced in mouse (Promega), and the LAB antibody was produced in rabbit and affinity purified (U.Nussbaumer and M.Affolter, unpublished). Secondary antibodies were conjugated either with alkaline phosphatase or with horseradish peroxidase (AB kit).

Electrophoretic mobility shift assays (EMSAs)

EMSAs were carried out as described (Chan *et al.*, 1996). The EXD protein was nearly full length (from amino acid 1 to 323) and fused to the His tag at its N-terminus in the pQE vector (Qiagen). LAB was from amino acid 158 to its C-terminus (Chan *et al.*, 1996). DFD protein was prepared as described in Chan *et al.* (1997). The oligos used for the mobility shift assays were annealed, and blunt-ended with radiolabeled nucleotides and Klenow polymerase. The sequence of the wild-type oligo was 5'caaatTGATGGATTGcccgcccg3'; the sequences of the mutant oligos are indicated in Figure 5. The amount of EXD used was always 100 ng per binding reaction; the two concentrations of LAB used were 10 and 80 ng, and the two concentrations of DFD used were 10 and 50 ng.

Acknowledgements

We would like to thank Mariann Bienz for discussion, and for generously providing many fly strains, as well as the lab550 enhancer. We also thank Konrad Basler for the pC β vector, and for helpful comments on this manuscript. We are indebted to Ute Nussbaumer for her generous help in generating transgenic fly strains, and to Fabrice Kiefer and Arsène Gschwind for invaluable computer assistance. This study was

supported by the Swiss National Science Foundation and the Kanton Basel-Stadt (M.A.), and by the NIH (R.S.M.). M.A. is a START fellow of the Swiss National Science Foundation and R.S.M. a Scholar of the Leukemia Society of America.

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Received on September 4, 1997; revised on October 2, 1997