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A HOX complex, a repressor element and a 50 bp sequence confer regional specificity to a DPP-responsive enhancer

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SUMMARY

A central theme during development and homeostasis is the generation of cell type-specific responses to the action of a limited number of extant signaling cascades triggered by extracellular ligands. The molecular mechanisms by which information from such signals are integrated in responding cells in a cell-type specific manner remain poorly understood. We have undertaken a detailed characterization of an enhancer that is regulated by DPP signaling and by the homeotic protein Labial and its partners, Extradenticle and Homothorax. The expression driven by this enhancer (lab550) and numerous deletions and point mutants thereof was studied in wild-type and mutant *Drosophila* embryos as well as in cultured cells. We find that the lab550 enhancer is composed of two elements, a Homeotic Response Element (HOMRE) and a DPP Response Element (DPPRE) that synergize. None of these two elements can reproduce the expression of lab550, either with regard to expression level or with regard to spatial restriction. The isolated DPPRE of lab550 responds extremely weakly to DPP. Interestingly, we found that the inducibility of this DPPRE is weak because it is tuned down by the action of a repressor element. This repressor element and an additional 50 bp element appear to be crucial for the cooperation of the HOMRE and the DPPRE, and might tightly link the DPP response to the homeotic input. The cooperation between the different elements of the enhancer leads to the segmentally restricted activity of lab550 in the endoderm and provides a mechanism to create specific responses to DPP signaling with the help of a HOX protein complex.

Key words: DPP, Signaling, Homeotic genes, Endoderm, Induction, Transcription, Gene regulation

INTRODUCTION

The progressive determination of cells by intercellular signaling cascades is a key feature of animal development. Recent studies have shown that a relatively small number of signal transduction pathways is involved in the correct temporal and spatial elaboration of the numerous different cell types present in complex multicellular organisms. The molecular mechanisms that control the specificity of the signaling response in different developmental contexts remain poorly understood. Indeed, it is still unclear how signaling through the same pathway leads to a variety of tissue- or segment-specific responses and how specificity is achieved.

The specificity of the response to members of the TGFβ/BMP superfamily of signaling molecules has been investigated in the past few years. TGFβ ligands control a large number of processes during development and homeostasis, and defects in TGFβ signaling cause a large number of pathologies. Distinct classes of TGFβ cytokines signal through similar pathways, involving heteromeric transmembrane Ser/Thr kinase receptor complexes at the cell surface and Smad family proteins as cytoplasmic effectors. A certain degree of specificity in the intracellular signal transduction cascade with respect to different TGFβ-like cytokines is reached at the level of the receptor-activated Smad proteins, dividing the ligands into two major subgroups, the BMP and the TGFβ/Activin subfamily. Nuclear factors that interact with receptor-activated Smad proteins have been isolated. However, the specificity of the response to members of the same subgroup and to the same ligand in different developmental contexts remains obscure (Hata et al., 2000; Massague and Chen, 2000; Massague and Wotton, 2000).

In *Drosophila*, the BMP 2/4 homologue Decapentaplegic (DPP) controls a large number of cell fate decisions during the development of the fly embryo (Raftery and Sutherland, 1999). It has been demonstrated that DPP signals through the same pathway in most of these processes, involving the receptors Punt (PUT) and Thickveins (TKV) and the Smad proteins Mother Against Dpp (MAD) and Medea (MED). The reiterated use of the same pathway in many different developmental decisions prompts the question of the specificity of the response to DPP signaling in various contexts. Although a complex of MAD and MED has been shown to bind to DPP target enhancers (Certel et al., 2000; Kim et al., 1997; Xu et

al., 1998), tissue-specific nuclear proteins that interact with the signal transducers have not yet been identified.

Cell type allocation in the *Drosophila* midgut can serve as a model system to study the specificity of the response to DPP signaling. DPP is expressed in two distinct domains of the visceral mesoderm – in parasegments (ps) 3 and 7; its expression is restricted to these domains by the action of genes of the HOM/HOX cluster (Bienz, 1994). DPP is secreted from visceral mesoderm cells and controls gene expression in specific domains, both in the visceral mesoderm and in the endoderm. Most prominently, DPP signaling from ps 7 induces the expression of the homeotic gene *labial* (*lab*) in a restricted domain of the endoderm, abutting the DPP expression domain in ps 7 of the visceral mesoderm (Bienz, 1997). However, DPP signaling from ps 3 does not lead to *lab* expression in the adjacent domain of the endoderm, and *lab* is also not induced in ps 3 and ps 7 of the visceral mesoderm. To elucidate the molecular mechanisms that control the DPP response in the midgut, we analyzed a 550 basepair (bp) enhancer from the *lab* gene, lab550, which is sufficient to drive expression in the domain of the endoderm abutting ps 7, in which endogenous LAB is expressed (Grieder et al., 1997; Tremml and Bienz, 1992). Our previous work has shown that the lab550 enhancer contains an essential, tripartite binding site for LAB and its co-factors Extradenticle (EXD) and Homothorax (HTH; Grieder et al., 1997; Ryoo et al., 1999). Strikingly, a short 45 bp element containing this site, termed the Homeotic Response Element (HOMRE), is able to drive by itself in a single copy substantial expression in the endoderm, in a pattern reminiscent of the endogenous *lab* gene; this expression is strictly dependent on the activity of the *lab*, *exd* and *hth* genes (Grieder et al., 1997; Ryoo et al., 1999). However, the lab550 enhancer only responds to DPP signaling when the 5′ located HOMRE is linked to downstream sequences that, by themselves, behave as a very weak DPP response element. Based on these findings, we proposed that the *lab* enhancer integrates both signaling and homeotic input and represents a model system to study interactions between these two important developmental regulators (Grieder et al., 1997; Mann and Affolter, 1998; Ryoo et al., 1999).

In order to determine the role of each subelement of lab550 to endodermal expression, we have carefully analyzed the activity of the lab550 enhancer and numerous deletions and point mutants thereof, in wild-type and mutant *Drosophila* embryos, as well as in cultured cells. Strikingly, we found that lab550 contained an extremely weak DPP response element (DPPRE), which seems to integrate both signal- and tissue-specific inputs; the inducibility of this DPPRE is weak because it is tuned down by the action of a repressor element. We also identified a 50 bp sequence element within the DPPRE that is absolutely essential for DPPRE and lab550 activity. The repressor and the 50 bp elements appear to be crucial for the cooperation of the HOMRE and the DPPRE, and might tightly link the transcriptional response to DPP to the presence of a homeotic protein complex. Our studies identify several elements that are required in concert to generate a local response to DPP in the endoderm. The further characterization of these elements might allow the elucidation of the molecular interactions that link homeotic and cell signaling information in responding cells.

MATERIALS AND METHODS

Isolation of the lab enhancer from *Drosophila hydei*

To find sequences related to the lab550 enhancer, we screened a genomic *D. hydei* library (a gift from D. Maier (Maier et al., 1990)) at low stringency with a radioactively labeled lab550 probe from *D. melanogaster*. Low-stringency hybridizations were performed overnight at 50-55°C in 6×SSC, 5×Denhardt's and 0.5% SDS, with salmon sperm DNA as carrier. Hybridization was followed by two 10 minute washes at room temperature and two 30 minute washes at hybridization temperature in 2×SSC, 0.5% SDS. We isolated several hybridizing phage clones; their homology was reconfirmed by low stringency Southern blotting with both a lab550 probe and a probe for the *lab* gene. Restriction analysis and low stringency Southern blotting allowed us to isolate a 664 bp fragment that was sequenced on both strands. Sequences of the lab550 (*D. melanogaster*) and H664 (*D. hydei*) enhancer fragments were aligned using ClustalX. The H664 fragment from *D. hydei* was also cloned by PCR into pCβ (see below) for transformation of *D. melanogaster*.

Fly stocks and transformants

Transformant lines were generated by standard procedures. 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. The endoderm-specific Gal4 line 48Y was obtained from N. Brown (Martin-Bermudo et al., 1997). The UAS-tkvQD (Nellen et al., 1996) and the UAS-armS10 (Zecca et al., 1996) lines were a gift from K. Basler; the UAS-rasV12 was obtained from Denise Montell (Lee et al., 1996). For the analysis of expression in mutant backgrounds, the following alleles were used: dpp^{s4}, $abd\text{-}A^{M1}$, lab^{VD1} and wg^{CX4}. The assay in *twi* mutants was carried out using a *twi* 48Y lab550 recombinant chromosome created by meiotic recombination. The presence of 48Y and lab550 was checked by Single-Fly PCR (Gloor et al., 1993), the presence of the *twi* allele by checking for homozygous lethality and embryonic phenotype.

Cloning and mutagenesis

All reporter constructs were generated using standard cloning procedures, and inserts were cloned into a *Kpn*I and *Bam*HI site of the nuclear *lacZ*-encoding P-element vector pCβ (a gift from K. Basler). Mutations and deletions of the original 550 bp *Cla*I *lab* fragment were generated with a PCR-based approach using the proof-reading polymerase Pfu. The construction of the original lab550-pCβ reporter, as well as the deletion variants 48/95-pCβ and 92/546-pCβ, have been described (Grieder et al., 1997). Other deletion constructs and the H664 construct were similarly amplified using primers starting at the indicated position and bearing an added *Kpn*I site at the 5′ end and an added *Bam*HI site at the 3′ end. The 550mCRE construct was created by subcloning the 550C fragment using primers containing the same restriction sites (Eresh et al., 1997) into pCβ. The point mutation in 550m300 was introduced by a two-step PCR amplification (Grieder et al., 1997). The sequence of the PCR primers can be provided on request. All constructs were sequenced using the Perkin Elmer Automated Sequencer ABI 320 and the AmpliTaq Big Dye Kit (Perkin Elmer). The MAD sites we mutated are indicated in Fig.1. In each case, two nucleotides were mutated in the consensus binding site (GCCGnCGC to GCTAnCGC); all sites identified on lab550 differ from the consensus at one or two positions. To generate the site 300 mutation, the stretch of five C residues starting at position 297 was mutated to ATATA; the construct lab550 from which all further m300 constructs were derived, contains two further mutations which were not associated with the derepression phenotype. The expression plasmids for Mad, Medea and Tkv^{QD} were a generous gift of Dr Kawabata. The lab550 enhancer was generated from a pBluescript plasmid containing the 550bp upstream regulatory region of the *lab* gene, cut with *Hin*dIII and *Xho*I, and cloned into the

luciferase vector pt81luc (Nordeen, 1988). All the other lab550 derivatives were generated from the corresponding pCβ vectors, cut with Asp718 and *Bam*HI, blunted with Klenow and cloned into pt81 luc opened with *Sma*I.

Antibody staining

The anti-β-Gal antibody was generated in mouse (Promega), and the LAB antibody was produced in rabbit and affinity purified (U. N. and M. A., unpublished). Secondary antibodies were conjugated either with alkaline phosphatase or with horseradish peroxidase, using the ABC kit (Vector labs). For confocal analysis, FITC- and Alexa-conjugated secondary antibodies were used (DAKO and Molecular Probes).

Transfection experiment

COS-1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum (Gibco), 100 units/ml of penicillin and 100 μg/ml streptomycin. Transfections were carried out by $CaPO_4$ precipitation. Typically, for a 6 mm dish, 10 μg of total DNA was added to cells that had reached 1/3 confluency. Forty-eight hours after transfection, cells were harvested for luciferase and β-gal activity as previously described (Vigano et al., 1998).

RESULTS

The lab550 HOMRE is necessary, but not sufficient, for lab550 expression in the endoderm

We have demonstrated previously that a short 45bp element at the 5′ end of the lab550 enhancer is required for lab550-driven reporter gene expression in the midgut endoderm. This element contains an essential binding site for the homeotic protein Labial (LAB) and its co-factors EXD and HTH and was therefore termed the Homeotic Response Element (HOMRE) (Grieder et al., 1997; Ryoo et al., 1999). As the nuclear translocation of EXD in the midgut endoderm has been shown to depend on DPP (and Wingless (WG)) signaling (Mann and Abu-Shaar, 1996), the lab550 HOMRE could well resume the observed dependence of lab550 on both, *lab* and DPP signaling. To investigate this possibility, we analyzed the temporal and spatial expression profile driven by lab550 and the 48/95 HOMRE in relation to endogenous *lab* expression. The sequence of the lab550 enhancer and the extent of the HOMRE (red arrows) are shown in Fig. 1A.

We monitored reporter gene expression at two different developmental stages and observed striking differences between the domains in which these two enhancers were active. At early stage 13, lab550-driven expression overlapped substantially with endogenous LAB protein (Fig. 1C). In contrast, expression driven by the HOMRE was clearly observed in two to three rows of cells posterior to the LAB expression domain at this stage and was not observed in the anterior LAB-expressing cells (Fig. 1D); in addition, the expression levels driven by the HOMRE were much lower than those driven by lab550 (data not shown). Note that we have previously shown that even in the cells in which we do not detect high levels of DPP-induced LAB, both constructs depend on *lab* function and we have argued that the low levels of LAB present in the endodermal primordia before fusion are critical for the expression (Grieder et al., 1997). At stage 14, during which the gut assumes the shape of a heart, the difference in the expression of the two constructs was maintained; lab550 was active in all cells expressing LAB (Fig. 1E-G), while HOMRE activity was not detected in the anterior

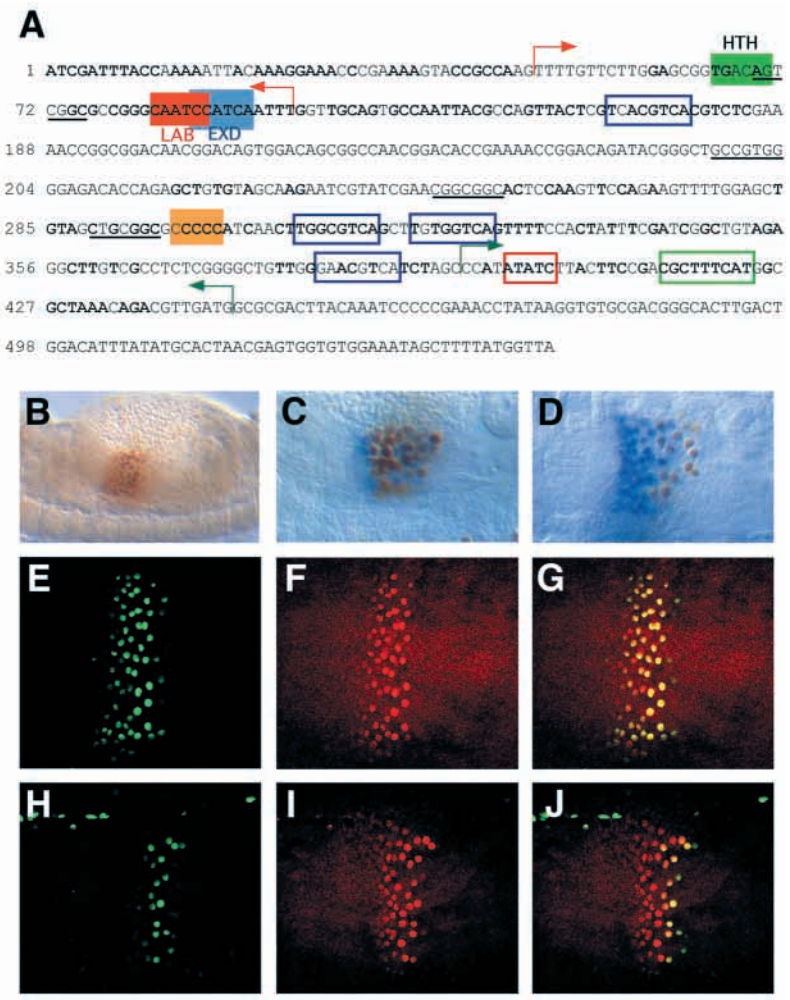

Fig. 1. The HOMRE does not activate transcription in the same domain as the entire lab550 enhancer. The sequence of the lab550 enhancer is shown in A. The HTH-, LAB- and EXD-binding sites are shown in green, red and blue boxes, respectively (Grieder et al., 1997; Ryoo et al., 1999). The extent of the 48/95 HOMRE is indicated by the red arrows and the extent of the 50 bp element (see text) by the green arrows. Four MAD/MEDEA-binding sites are underlined and four CREs are boxed in blue. The repressor element shaded in orange. A GATA site is boxed in red and a HMG-binding site in green. Nucleotides conserved between the *D. melanogaster* lab550 enhancer and the corresponding element from *D. hydei* are printed in bold; non-conserved residues are not bold. The sequence stretch from 185 to 215 is not conserved between the two species. (B-D) The expression of endogenous lab (brown, B) is compared with the expression driven by lab550 (C; lab in blue, lab550 driven β-GAL expression in brown) and the expression driven by the HOMRE 48/95 (D; lab in blue, HOMRE in brown) in stage 13 embryos. Clearly, lab550 mimics the expression of lab, whereas HOMRE-driven expression is mostly posterior to lab. (E-J) Using confocal microscopy, the expression of lab550 (green; E,G) and the HOMRE (green; H,J) is compared with endogenous lab (red; F,G,I,J) in stage 14 embryos. Expression levels were higher in later stages and it was not possible to use confocal microscopy in stage 13 embryos.

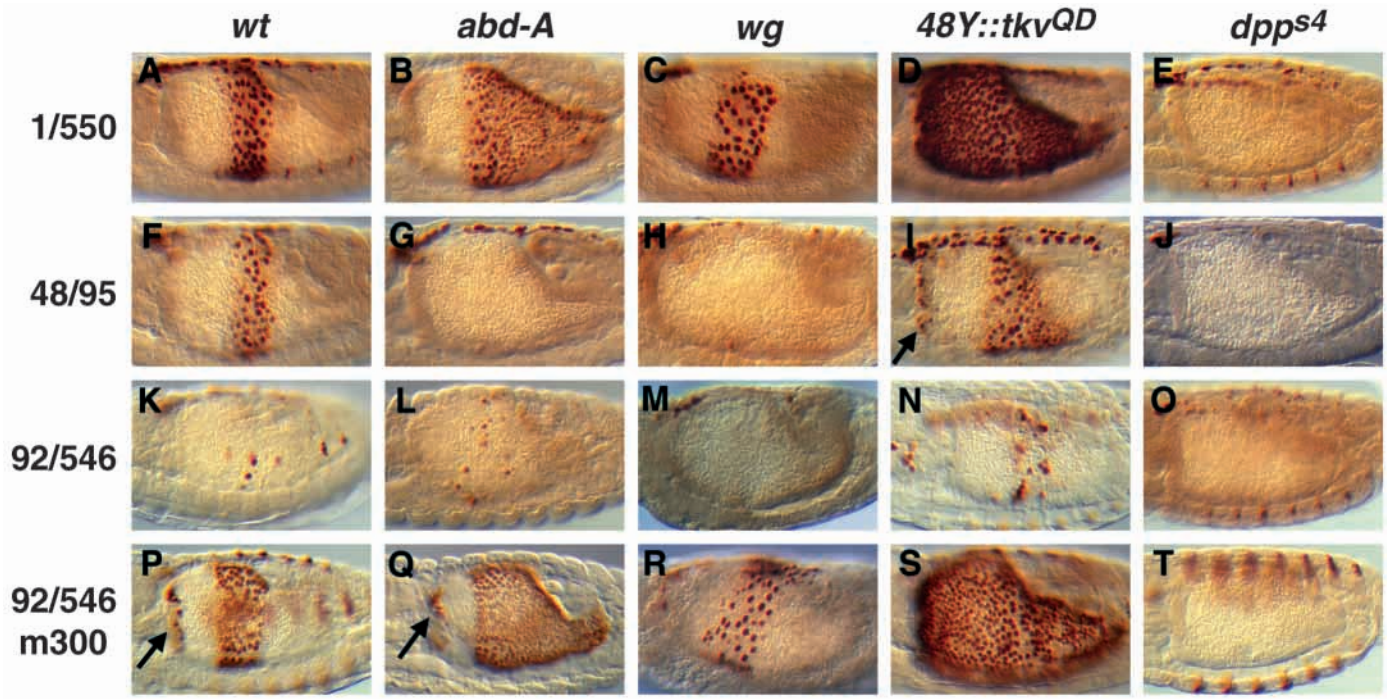

Fig. 2. Neither the HOMRE nor the DPPRE shows the same genetic requirement for expression as lab550. Expression of lab550 (A-E) is compared with the expression of 48/95 (HOMRE; F-J), 92/546 (DPPRE; K-O) and 92/546m300(DPPREm300; P-T). Expression is analyzed in wild-type embryos (A,F,K,P) or in embryos mutant for *abd-A* (B,G,L,Q), *wg* (C,H,M,R) or dpp^{s4} (E,J,O,T). Expression was also analyzed in embryos expressing a constitutive active version of the DPP receptor TKV (tkv^{QD}) in the endoderm (D,I,N,S). Note that in contrast to lab550, 92/546m300 is strongly expressed in the gastric caeca endoderm (arrows, P,Q).

rows of these cells but clearly present in cells posterior to the endogenous *lab* domain (Fig. 1H-J). We conclude that although the expression pattern of the lab550 and the HOMRE reporter partially overlap, the HOMRE clearly does not account on its own for the activity of lab550 or for the expression of the endogenous *lab* gene. Rather, HOMRE expression mimics the expression of an oligomerized LAB/EXD site in late embryos (rp3; Popperl et al., 1995); similar to the HOMRE, rp3 is active both in the second and third midgut convolution (data not shown), but in contrast to the HOMRE, rp3 is already active in the posterior endodermal primordia before fusion (Chan et al., 1996; Popperl et al., 1995).

To better understand the reasons causing the different expression domains driven by lab550 and the HOMRE, we analyzed the activity of these elements in different genetic backgrounds that modify the levels and extents of DPP signaling in the midgut. As we have reported previously (Grieder et al., 1997), lab550 follows the posterior expansion of *dpp* expression in the visceral mesoderm of *abd-A* mutants (Fig. 2B), whereas the HOMRE is completely inactive in *abd-A* mutant embryos (Fig. 2G). As *abd-A* does not affect endoderm development before induction occurs, we conclude that the HOMRE does not respond to ectopic Dpp (see also below).Therefore, HOMRE activity displays a strict requirement for an additional visceral mesodermal factor missing in *abd-A* mutants. As *wg* expression is absent in ps8 of the visceral mesoderm in *abd-A* (Immergluck et al., 1990), we directly monitored the activity of the two enhancers in *wg* mutants. The expression driven by lab550 was only slightly reduced (Fig. 2C), most probably owing to the reduced levels of *dpp* signaling in *wg* mutants (Yu et al., 1996). Strikingly, the HOMRE drove no expression in the absence of *wg* (Fig. 2H). Therefore, an additional input from the WG signaling cascade is necessary for the activity of the HOMRE, but is not essential

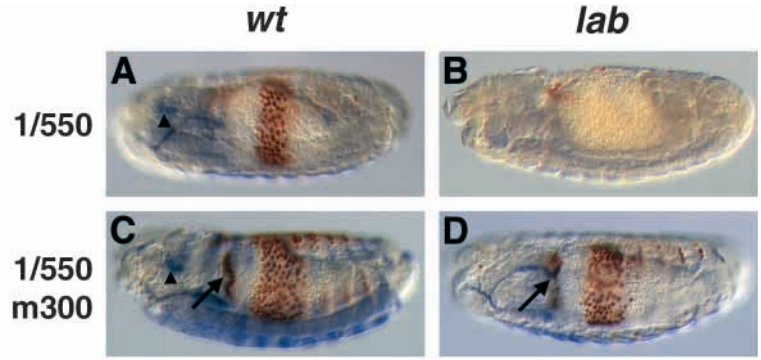

Fig. 3. The mutated DPPRE is independent of the activity of the HOX complex. Expression of lab550 (A,B) is compared with the expression of lab550m300 (C,D) both in wild-type (A,C) and *lab* mutant embryos (B,D). Embryos were double stained for β-gal activity driven by the enhancers (brown) and LAB protein (blue). The mutated element is strongly active in *lab* mutants both in ps 7 and in the gastric caeca (arrow). Note that the diagnostic LAB staining in the CNS (arrowheads) is missing in the mutants.

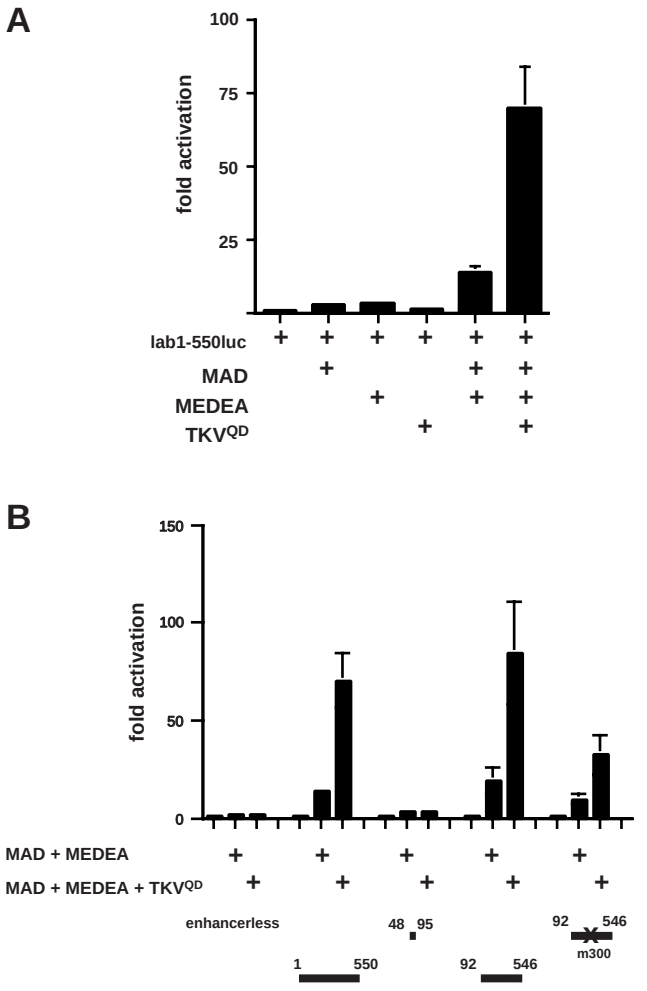

Fig. 4. 92/546 acts as a strong DPP response element in COS cells. The expression of luciferase under the control of the lab550 enhancer or derivatives thereof was analyzed in COS cells after co-transfection with combinations of MAD-, MEDEA- and TKV^{QD}-expressing plasmids. Cells were transfected with 4 μg of the reporter plasmids lab1-550luc (A) or its derivatives, schematically represented at the bottom of the graph, and (B) co-transfected with 2 μg of each indicated expression plasmids. enhancerless: parental luciferase plasmid pt81-luc. The amount of transfected DNA was kept constant (10 μg) by addition of psG5 plasmid. Bars represent the luciferase activity of transfected cell extracts (mean±s.e.m. of three to ten independent experiments, each carried out in duplicate), expressed as -fold activation over the basal activity of the reporter construct. Values were normalized by co-transfection of 0.1 μg of a pCMV-β-gal plasmid as an internal standard. lab550 activity is increased 70-fold after the co-transfection of all four plasmids. (A) Although MAD and MEDEA together also stimulated expression of lab550 (14-fold), the addition of TKV^{QD} further increased expression fivefold; TKV did not stimulate lab550 expression in the absence of co-transfected MAD and MEDEA. In contrast to lab550, the activity of HOMRE (B) was increased only threefold by DPP signaling. DPPRE activity was increased 84-fold and the activity of DPPRE carrying mutation 300, which allowed for a strong DPP response in the endoderm, was increased 32-fold. Thus, mutation 300 did not show a stimulatory effect in COS cells but rather somewhat reduced the DPP response. The reduced induction of 92/546m300 when compared with 92/546 was due to a higher basal level of 92/546; the measured activity levels after induction were similar (data not shown).

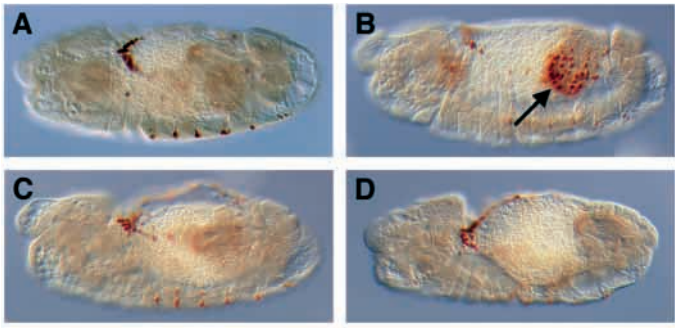

Fig. 5. lab550 can be induced by DPP signaling in the absence of the visceral mesoderm. In a *twi* mutant embryo, expression of the lab550 construct is absent in the endoderm (A). The endodermal expression driven by lab550 can be restored in *twi* mutants upon ectopic expression of an activated form of the TKV receptor, tkv^{QD}, under UAS control, using the endoderm-specific GAL4 line 48Y (B, arrow). When ectopic WG or MAPK signaling were induced in a similar, cell-autonomous, way in *twi* mutants, using 48Y and an activated form of arm, arm^{S10} (C), and of Ras, ras^{V12} (D) under UAS control, endodermal expression of lab550 was not restored.

for lab550 activity. To confirm these results, we also monitored the activity of the two enhancers upon the expression of high levels of the activated DPP receptor Tkv^{QD} (Nellen et al., 1996) in the endoderm by using the endoderm-specific driver 48Y (Martin-Bermudo et al., 1997). lab550-driven expression was expanded and strong throughout most of the endoderm (Fig. 2D). HOMRE-driven expression was only slightly expanded posteriorly and showed weak ectopic expression in the gastric caeca endoderm (Fig. 2I, see arrow). Interestingly, these two sites of ectopic endodermal expression of the HOMRE abut WG-expressing cells of the visceral mesoderm (anterior to the gastric caeca and in ps 8) and therefore could reflect the strict requirement of HOMRE for WG signaling.

These results demonstrate clearly that the HOMRE does not represent a minimal enhancer mimicking lab550 driven expression, as it displays both different spatial activity as well as different genetic requirements for activity. Most importantly, the HOMRE does not account for the DPP responsiveness of lab550.

The lab550 enhancer harbors an attenuated DPPRE in its 3′ region

As the HOMRE only partially accounts for the activity of the lab550 enhancer, we looked for additional elements that contribute to the regulation of lab550. We have previously proposed that the activity of lab550 is a consequence of a synergistic interaction between the HOMRE and the remaining 3′ sequences of lab550, which are thought to integrate DPP signaling (Grieder et al., 1997). However, the 3′ region of the lab550 enhancer, termed 92/546, drives expression in only a few cells (Fig. 2K), which makes it difficult to assess its DPP responsiveness. Indeed, only few cells display 92/546-driven reporter gene expression in the posterior endoderm region of *abd-A* mutants (where *dpp* is strongly expressed in the entire posterior visceral mesoderm), in sharp contrast to the strong posterior expansion found for lab550 (compare Fig. 2L with Fig. 2B; Grieder et al., 1997). However, the 92/546 enhancer fragment showed a weak but substantial response to high levels

of DPP signaling (Fig. 2N). These results point to the existence of a weak DPP response element located in the 3′ part of the lab550 enhancer.

As a result of a mutational analysis of in vitro binding sites for the nuclear factor Schnurri (Grieder, 1997; Grieder et al., 1995), we identified a site around position 300 of the enhancer that, when mutated (see Materials and Methods), resulted in a dramatic change in the activity of the enhancer. A reporter construct containing this mutation, termed 92/546m300, showed a striking change, both qualitative and quantitative, in its response to DPP signaling in the endoderm. Whereas 92/546 was only weakly active in a few cells, 92/546m300 was highly active and substantially expanded adjacent to ps 7. Strikingly, expression was also clearly detected in a domain abutting ps 3 of the visceral mesoderm, where *dpp* is also expressed (Fig. 2P, arrow). Strong expression was maintained in the four gastric cacae in later stages (data not shown). Moreover, the expression driven by 92/546m300 was posteriorly expanded in *abd-A* mutants and strongly detected throughout the entire endoderm in embryos expressing tkv^{QD} under the control of the endodermal driver 48Y (Fig. 2Q,S). The fact that the activity of this mutated element is absent in dpp^{S4} mutants (Fig. 2T) but hardly affected in *wg* mutants (Fig. 2R) underscores the strong DPP responsiveness of the 92/546m300 enhancer. The enhancer remained endoderm-specific, as ubiquitous expression of tkv^{QD} resulted in ectopic expression in the endoderm only (data not shown). These experiments demonstrate that the weak DPP-responsive element (DPPRE) in the 3′ part of the lab550 enhancer (92/546) is the result of the presence of a repressor element around site 300 in an otherwise very sensitive, endodermal DPP-responsive enhancer.

As the 3′ DPPRE with the site 300 mutation is strongly DPP responsive in endodermal cells adjacent to ps 3 and ps 7, and lacks the HOMRE, we expected that the introduction of the same mutation in the full-length lab550 would result in an enhancer that loses its dependence on *lab* function for expression. Indeed, lab550m300 is strongly activated in the anterior and central midgut endoderm by DPP in *lab* mutants (Fig. 3D); the same is true for 92/546m300 (data not shown). This is in sharp contrast to the expression driven by lab550, which lacks the site 300 mutation; expression of this enhancer is strongly reduced in the absence of *lab* (Fig. 3B; Grieder et al., 1997). These experiments show that the mutation of site 300 abrogates the need of homeotic input for the DPP-dependent activity of the lab550 enhancer.

The DPPRE is strongly induced by DPP signaling in cultured cells

To confirm more directly the responsiveness of lab550 and some of its subelements to DPP signaling, we co-transfected COS cells with different reporter constructs and components of the DPP signaling pathway, including the activated receptor TKV^{QD}, MAD and MEDEA. The rationale behind these experiments was to assess whether the enhancer elements would respond to DPP signaling in this heterologous system; such a response could much more comfortably be interpreted as a direct activation of the enhancer by DPP signaling mediators, in contrast to an indirect activation via the induction of secondary signals or via the induction of transcriptional regulators that in turn, activate the enhancers.

Indeed, we found that the full-length lab550 enhancer could be activated in the presence of co-transfected TKV^{QD}, MAD and MEDEA (Fig. 4A). No activation was seen in the presence of the activated receptor alone. In the presence of MAD and MEDEA only, a 14-fold stimulation was observed; this activity was synergistically enhanced in the presence of the activated receptor, leading to a 70-fold increase in luciferase levels (Fig. 4A). Clearly, lab550 represents a DPP responsive enhancer in COS cells upon the reconstruction of the DPP signaling system in these heterologous cell system. This allowed us to define the sequences necessary for the (most likely) direct signaling response in this heterologous system and compare the results with those obtained in *Drosophila* embryos.

A reporter construct containing the HOMRE showed only very little activity above basal levels after transfection with TKV^{QD} and the Smad-encoding plasmids (Fig. 4B). This result is consistent with our findings that the HOMRE does not respond well to ectopic DPP in the *Drosophila* embryo. However, the same transfection regime led to a strong and reproducible 70-80-fold induction of the 92/546 reporter constructs (Fig. 4). The fact that lab550 and its subelement 92/546 displayed similar levels of reporter gene activation upon signaling demonstrated that most, if not all, of the DPP-responsive sequences are located on the 3′ fragment of the lab550 enhancer; this is in agreement with our findings in the *Drosophila* embryo (see above). These results unambigously show that the lab550 enhancer harbors a DPPRE in its 3′ part. Moreover, this DPPRE is likely to be inhibited in its activity or inducibility in *Drosophila* embryos by the binding, at site 300, of a factor that is present throughout the *Drosophila* endoderm; this factor(s) appears to be absent in COS cells, as the introduction of mutation 300 did not increase the DPP responsiveness of lab550 or 92/546 (Fig. 4B; see also Figure legend).

Signaling through the DPP pathway is necessary and sufficient to activate lab550

Our cell culture experiments and our in vivo studies that analyze *dpp* loss-of-function and *dpp* gain-of-function situations (ectopic *dpp* expression in *abd-A* mutants and ectopic expression of an activated DPP type I receptor) clearly demonstrate that the lab550 enhancer can be strongly induced by DPP signaling. This result contrasts somewhat with previous work that has proposed the activity of lab550 to result from a functional intertwining of DPP signaling with Epidermal Growth Factor (EGF) signaling in the endoderm (Szuts et al., 1998). In addition, previous reports have shown both positive and negative effects of WG signaling from the visceral mesoderm on the expression of *lab* (Hoppler and Bienz, 1995).

To assess the relative importance of the DPP and the other signaling pathways in *lab* induction and/or in lab550 regulation, we expressed activated components of each of these pathways in a cell-autonomous manner in the endoderm in the absence of the inducing tissue – the visceral mesoderm. For this purpose, the visceral mesoderm was genetically ablated using mutants for the mesoderm-determining factor *twist*; in these mutants, the visceral mesoderm does not develop, but endodermal cell fates are determined. In *twi* mutant embryos, the lab550 enhancer fails to be activated in the endoderm (Fig. 5A), as predicted from the lack of the inductive signal(s).

Specific signaling pathways were activated in the endoderm of *twi* mutants in a cell-autonomous fashion using either a constitutively active DPP receptor, TKV^{QD}, which triggers the DPP signaling pathway, an active form of ARMADILLO, ARM^{S10}, which triggers the WG signaling pathway, and the active form of RAS, RAS^{V12}, which triggers the MAPK pathway. In *twi* mutant embryos, overexpression of ARM^{S10} and RAS^{V12} had no effect on the activity of lab550 (Fig. 5C,D, respectively). In contrast, overexpression of TKV^{QD} led to a strong induction of lab550-driven reporter gene expression (Fig. 5B). This activation of lab550 is mediated by the DPPRE, as a construct containing the 92/546m300 element was also strongly activated by DPP signaling in *twi* mutants (data not shown). We also monitored the expression of the endogenous *lab* gene in *twi* mutant embryos that expressed the activated components of the aforementioned pathways. Consistent with the results obtained for the lab550 enhancer, LAB protein could only be detected in the endoderm of *twi*- embryos when TKV^{QD} was concomitantly expressed in the same germ layer (see also Grieder et al., 1995); expression of ARM^{S10} and RAS^{V12} had no activating effect (data not shown). Combined with the cell culture assays (Fig. 4), these experiments demonstrate that DPP signaling is sufficient to strongly activate lab550 in the absence of other signals from the visceral mesoderm.

Both MAD and CRE-binding sites contribute to the DPP response of lab550

Since the 92/546 DPPRE recapitulates most if not all of the DPP responsiveness of the lab550 enhancer, we sought to determine which sites on this subfragment mediate this response. We searched the lab550 enhancer for binding sites for MAD, which has been shown to bind DPP target enhancers (Kim et al., 1997; Xu et al., 1998). We found four sites (at positions 80, 210, 240 and 290; see Fig. 1) that closely match the consensus binding site for MAD (GCCGnCGC); these binding sites bound purified MAD protein with the same affinity as previously published sites on the vg^{Q} or the *tin* enhancer (data not shown; Kim et al., 1997; Xu et al., 1998). We mutated all the sites individually and in combination. Somewhat surprisingly, mutating all four MAD binding sites led to only a moderate reduction in expression of both the lab550 construct and of the activated DPPRE construct, 92/546m300 (Fig. 6C,F). However, these results are in agreement with previous studies that showed only a slight reduction in lab550 activity upon mutation of two MAD-binding sites (Szuts et al., 1998). To better quantify the effect of mutating the potential MAD sites on lab550, we assayed the activity of this mutated form of lab550 in cell culture experiments. In this assay system, we observed a clear reduction in the response to DPP signaling of the mutated enhancer compared with its wild-type form (Fig. 6A). Therefore, we conclude that these MAD-binding sites contribute to the DPP responsiveness of lab550, both in vivo and in cell culture experiments; however, other sites must also confer DPP responsiveness.

It was previously reported that binding sites resembling CREs are necessary for the full activity of lab550 (Eresh et al., 1997; Szuts et al., 1998). We therefore assayed the effect of mutating those sites on the activity of the lab550 enhancer and of the activated DPPRE, both in embryos and in tissue culture. Whereas the expression driven by lab550 was only slightly reduced upon mutation of all four CRE sites, the derepressed DPPRE (92/546m300) was strikingly inactive, showing expression in a few cells only (Fig. 6G). In addition, cell culture assays with the CRE-mutated form of lab550 show a strongly reduced response to DPP of lab550mCRE (Fig. 6A). It has been proposed that the bZIP transcription factor Dfos could directly or indirectly mediate part of the response of *lab* to DPP (and EGF) signaling in the endoderm by binding on the lab550 enhancer (Riese et al., 1997; Szuts and Bienz, 2000). To test this possibility, we assayed the expression of

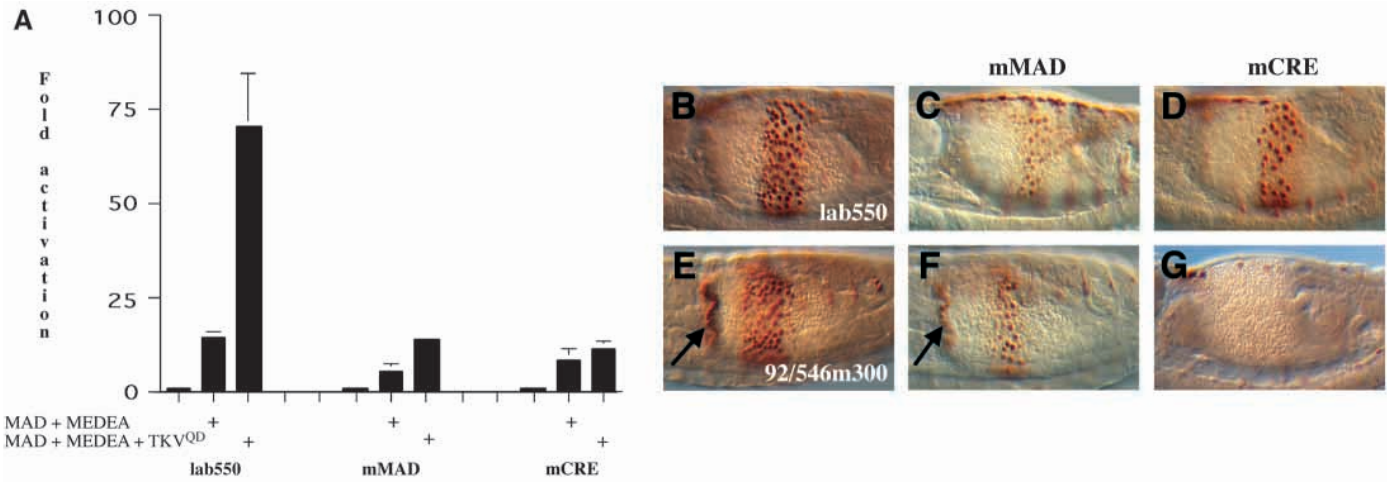

Fig. 6. MAD and CRE sites are important for the DPP response, both in the embryo and in COS cells. (A) Luciferase activity of the lab550 enhancer and two mutant derivatives upon stimulation of DPP signaling in transfected COS-1 cells. The DPP response is expressed as -fold activation over the basal activity of each reporter plasmid alone, which is indicated at the bottom of the graph (see the legend of Fig. 4 for details). The introduction of the four MAD mutations and the introduction of the four CRE mutations lead to a reduction in the response to DPP signaling from 70-fold to 14-fold and 11-fold, respectively. In the embryo, lab550-driven expression (B) was reduced by the introduction of mutations in either the MAD sites (C) or the CRE sites (D). 92/546m300-driven expression (E) was also reduced by mutations in the MAD sites (F) and was hardly detectable after mutating the CRE sites (G).

reporter constructs containing the activated DPPRE in *kayak* (*kay*) mutants, which show no zygotic Dfos expression. We found that the expression driven by 92/546m300 was reduced in cells adjacent to ps 7 but not adjacent to ps 3 (data not shown). Unfortunately, embryos from germline clones could not be analyzed because they do not complete oogenesis (Ernst Hafen, personal communication). Nevertheless, these findings suggest that Dfos mediates part of the response to DPP signaling of lab550 adjacent to ps 7 (maybe by binding on the CRE sites found on the DPPRE subfragment; see Szuts and Bienz, 2000), but that other factors must be able to substitute for Dfos in the gastric caeca.

A 50 bp element in the DPPRE is essential for its DPP inducibility

To further determine in a more unbiased and comprehensive manner the sequence elements that are necessary for lab550 expression and its response to DPP signaling, we undertook a systematic deletion analysis of the DPPRE. To overcome the problem of the very weak expression of the wild-type DPPRE, 92/546 (Fig. 2K), we made use of its highly active mutant form, 92/546m300 (Fig. 2P), to monitor the loss of response upon deleting sequences from both the 5′ end (at position 92) and the 3′ end (at position 546) of the DPPRE (see Figs 7 and 8).

Deletion of sequences from the 5′ end did not result in a substantial reduction of expression until position 193 was reached; the removal of additional sequences led to a progressive reduction in activity, resulting in complete loss upon deletion of sequences 5′ of position 344. Note that all three MAD binding sites found on the DPPRE are clustered between position 210 and 300, underscoring the contribution of these sites to the DPP responsiveness of lab550. Deletion of sequences from the 3′ end revealed the existence of an absolutely essential 50 bp element located between position 394 and 444 (Fig. 7); whereas deletion up to position 444 only resulted in a slight reduction of expression (Fig. 8A), deletion up to position 394 abolished expression altogether (Fig. 8D).

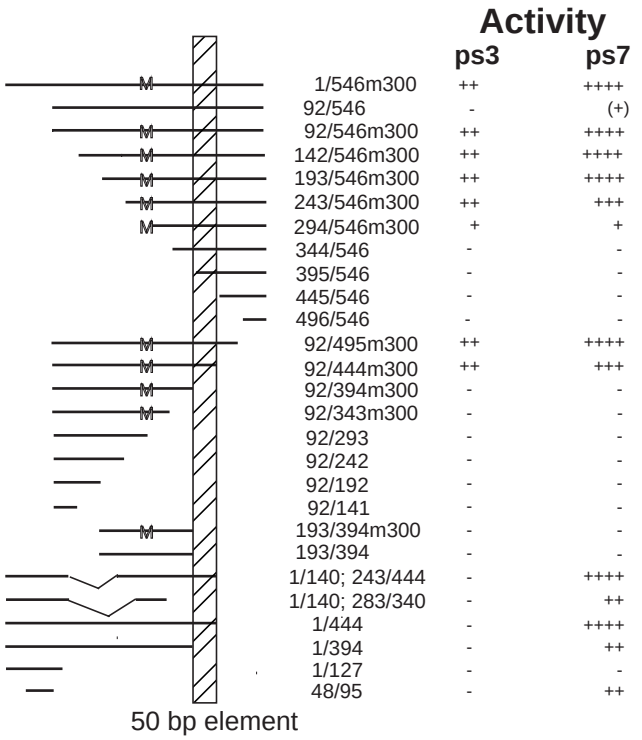

Fig. 7. Extensive deletion analysis defines an essential 50 bp element A large number of deletion constructs were analyzed for expression in the embryo. M stands for the presence of the mutation at site 300, which leads to a dramatic increase in the sensitivity of the enhancers to DPP. Clearly, deletion of sequences between 444 (1/444m300) and 394 (1/394m300) (deletion of the 50 bp element) leads to a loss of expression in endodermal cells adjacent to ps 3 and ps 7. The equivalent deletion was also analyzed in the context of lab550.

The 50 bp element is essential for the activity of lab550

The essential 50 bp element was identified in the context of a derepressed DDPRE (92/546m300). To assess the contribution of the 50 bp element (consisting of the sequences from 394 to 444) to the activity of the lab550 enhancer, we assayed the expression of a reporter construct containing the sequences between position 1 and 394 (1/394; Fig. 8E) and compared it with a reporter carrying the 50bp longer version, 1/444 (Fig. 8B). We found that whereas 1/444 displayed a pattern of expression similar to lab550, the expression driven by 1/394

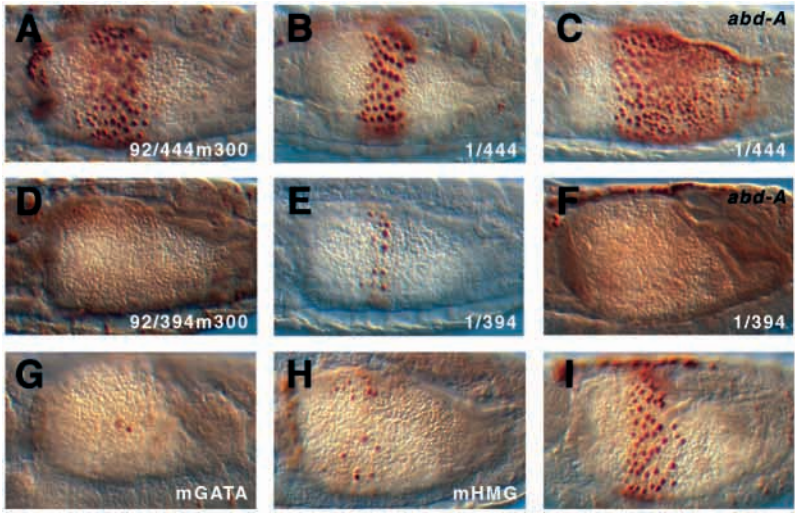

Fig. 8. The 50 bp element is required for lab550 activity. 92/394m300 was not active in wild-type embryos (D), while the same enhancer containing the 50 bp element (92/444m300) was active both in the endoderm of the gastric caeca and adjacent to ps7 (A). Mutation of either the GATA- (G) or the HMG- (H) binding site in 92/546m300 resulted in strongly reduced expression. In the absence of the mutation in the putative repressor binding site 300 and in the presence of the HOMRE, expression of the construct that lacked 50 bp (1/394) was weak in wild-type embryos (E) and inactive in *abd-A* mutants (F). Expression driven by 1/444 was strong in wild-type embryos (B) and expanded in *abd-A* mutants (C). Expression of a minimal enhancer construct (1/140;243/444) was also strong in wild-type embryos (I) and strong and posteriorly expanded in *abd-A* mutants (data not shown).

resembles more closely the HOMRE-driven expression (Fig. 8E; compare with Fig. 1D,H). To assess the DPP responsiveness of each of these constructs, we analyzed their expression in an *abd-A* mutant background in which *dpp* is expressed throughout the posterior visceral mesoderm (see Fig. 2). Strikingly, the 1/394 construct was inactive in *abd-A* mutants (Fig. 8F), whereas the 1/444 (Fig. 8C) construct drove strong and expanded expression similar to lab550. This demonstrates that the DPPRE is inactivated upon deleting sequences between 394 and 444, and demonstrates a clear requirement for this 50 bp element with regard to the DPP responsiveness and/or the overall activity of lab550.

To get a first glance at whether the 50 bp element might be a direct target for DPP signaling mediators or might, for example, be an element that provides tissue specificity, we analyzed its behavior in cell culture. The activity of the 1/444 enhancer is increased 36-fold by DPP signaling, and the deletion of the 50 bp element that is crucial for inducibility in embryos results in a slight reduction in DPP inducibility (but the activity of the 1/394 enhancer was still increased 20-fold by DPP signaling; Fig. 9). We also tested the 50 bp element directly for its ability to respond to DPP signaling in the cell culture assay. We found that the activity of this element was weakly but reproducibly increased 10-fold by DPP signaling. Although the element does not contain any recognizable MAD/MEDEA-binding sites (see Fig. 1), it appears to be able to sense DPP signaling in COS cells and is strictly required for DPP inducibility in *Drosophila* embryos.

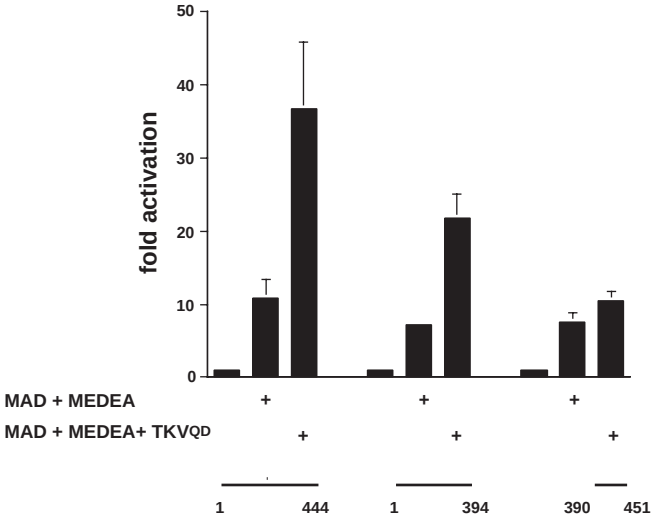

Fig. 9. The 50 bp element is required for full DPP responsiveness in COS cells. Luciferase activity of the lab550 enhancer and two 3′ deleted derivatives upon stimulation of DPP signaling in transfected COS-1 cells. The DPP response is expressed as -fold activation over the basal activity of each reporter plasmid alone, which is schematically represented as a bar with the nucleotide position of the deletion at the bottom of the graph (see the legend of Fig. 3 for details). Deletion of the 50 bp element reduced the induction of 1/394 (22-fold) compared to 1/444 (37-fold) in COS cells. The 50 bp element (a slightly extended version was put into the expression vector for cloning purposes) is induced 10-fold by the activated TKV receptor and the co-transfected Smad-expressing plasmids.

The 50 bp element contains essential GATA- and HMG box-binding sites

In order to achieve more insight into possible regulators acting on the 50 bp element, we tried to narrow down its requirement to individual binding sites; we therefore isolated the corresponding *lab* enhancer from a distantly related *Drosophila* species, *Drosophila hydei*. An element of 664 bp was isolated that shows a high sequence homology to lab550 (see Fig. 1). This element, termed H664, is able to drive an expression pattern very similar to that driven by lab550 in transgenic *D. melanogaster* (data not shown). The extent of the sequence similarity is highest in the HOMRE, with the LAB/EXD site fully conserved, whereas the HTH site shows only limited conservation. Moreover, a 5 bp sequence block is conserved around site 300, as well as three out of the four CRE sites, underscoring the importance of all of these sequences for the activity of lab550. We found only two conserved sites in the 50 bp between position 394 and 444: one closely resembles a GATA-binding site and the other bears some homology to a binding site for proteins of the HMG group, such as TCF and SOX. SOX proteins have been shown to act as tissue-specific factors (Kamachi et al., 2000; Pevny and Lovell-Badge, 1997) and GATA proteins are essential for endoderm formation both in insects and vertebrates (Zaret, 1999). Mutation of the GATA site only weakly reduced the activity of lab550, but led to a significant reduction of activity of the activated DPPRE, 92/546m300 (Fig. 8G). The expression of both lab550 (data not shown) and 92/546m300 (Fig. 8H) were clearly reduced upon mutation of the HMG-binding site. These experiments suggest that lab550 activity requires the binding of factors from the HMG and the GATA family to specific sites in an essential 50 bp element found in the 3′ part of the lab550 DPPRE.

The lab550 subelements act synergistically to drive expression in the endoderm

Based on the findings reported above, we constructed a minimal enhancer that should resume all the properties of the full-length lab550 enhancer. Accordingly, we fused a fragment that encompassed the large extent of sequence conservation around the HOMRE (1 to 140; see Fig.1) to a fragment extending from 243 to 444, thereby containing the conserved region around position 300, the MAD and CRE sites and the tissue-specific 50bp element described above (see Fig. 1). Such an element was able to drive a pattern of expression similar to that driven by lab550 (Fig. 8I); moreover, it showed the same genetic properties as the lab550 enhancer: the expression driven by this minimal enhancer was expanded posteriorly in *abd-A* mutants and throughout most of the endoderm upon ectopic activation of the DPP pathway in this tissue (data not shown).

Strikingly, none of the subfragments used to construct this minimal enhancer was able to drive substantial expression on its own. To our surprise, when we tested the activity of a fragment containing the conserved sequences around and including the HOMRE (1/127; see Fig. 1), we found that such an element was unable to drive expression in the endoderm, despite the presence of a functional HOMRE (Fig. 7). This could be due to the binding of a repressor(s) to the stretch of conserved sequence between 95 and 140. An element centered around position 300 (193/394), which contained three CRE and

three MAD sites and the repressor site, also failed to display any activity, even upon mutation of the repressor site (see Fig. 7). Finally, in the course of our deletion analysis of the activated DPPRE (92/546m300), we have tested two constructs, 344/546 and 395/546, that contain the 50 bp element; these enhancer fragments were completely inactive (see Fig. 7). We conclude that none of the functional elements of lab550 is able to drive significant DPP-dependent expression in vivo; only the combination of the subfragments, as found in the minimal enhancer, is able to resume the properties of the lab550 enhancer. Therefore, the lab550 enhancer is composed of several elements, or modules, that interact to provide spatial restriction, tissue specificity and signal inducibility, resulting in the proper activation of lab550 in a restricted domain of the endoderm that underlies ps 7 of the visceral mesoderm.

DISCUSSION

A central theme during development and homeostasis is the generation of cell type-specific responses to the action of a limited number of extant signaling cascades triggered by extracellular ligands. The molecular mechanisms by which information from such signals are integrated in responding cells in a cell type-specific manner remain poorly understood. Major progress has recently been made with regard to signals triggering receptor tyrosine kinases (RTKs; Flores et al., 2000; Halfon et al., 2000; Xu et al., 2000). It has been demonstrated that much of the specificity seen in nuclear responses of cells to RTK activation is generated by combining a generic RTK signaling pathway with inputs both from other signaling pathways and from pre-existing cell- or tissue-specific transcription factors. Here, we show that the specificity of the nuclear response to DPP signaling in the developing *Drosophila* endoderm results from the cooperation between effectors of DPP signaling binding on a DPPRE and a homeotic protein complex binding on a genetically linked HOMRE. Importantly, we find that the activity of the DPPRE is tuned down in the endoderm by a repressor element, which hinders the activation of DPPRE by DPP alone, thus allowing the HOMRE to synergize with the DPPRE in a restricted central portion of the midgut endoderm. Therefore, an interplay, on a single enhancer, between homeotic proteins, DPP signaling mediators and a repressor can determine the transcriptional response to DPP signaling in the *Drosophila* midgut.

The segment-specific response of lab550 to DPP signaling is controlled by the interaction of the HOMRE with the DPPRE

Our previous analysis of the endodermal enhancer of the DPP target gene *lab* has uncovered an essential binding site for the homeodomain protein LAB and its co-factors EXD and HTH in the lab550 HOMRE (Grieder et al., 1997; Ryoo et al., 1999). The HOMRE in the *lab* enhancer represents the only *Drosophila* regulatory element identified so far to which a defined HOX protein (LAB) binds in conjunction with the two well-characterized HOX partners, HTH and EXD. Thus, this element can serve as a paradigm to study how HOX protein complexes regulate gene expression in vivo.

Here, we demonstrate that lab550 contains a (repressed) DPPRE that is genetically separable from the HOMRE but functionally linked to the latter. The DPPRE integrates two different inputs in two modules. One module of the DPPRE is composed of a repressor site, flanked by MAD-binding sites on one side, and by CRE-binding sites on the other. The role of the CRE and MAD sites in the DPP-responsiveness of lab550 has already been demonstrated (Eresh et al., 1997; Szuts et al., 1998) and is further supported by our own findings, both in the embryo and in cell culture (see Fig. 6). The second module, the 50 bp element, could represent a tissue-specific DPP signaling module. All constructs (lab550, 92/546 and 92/546m300) are tissue specific and are only induced by DPP signaling in the endoderm. The essential 50 bp (394/444) element we identified in the 3′ part of the DPPRE contains conserved potential binding sites for a GATA factor and for a member of the HMG family. GATA factors play a central role during endoderm formation throughout animal evolution (Zaret, 1999). Three GATA factor-encoding genes are known in *Drosophila*, *serpent* (*srp*), *pannier* (*pnr*) and *grain* (*grn*; Brown and Castelli-Gair Hombria, 2000; Ramain et al., 1993; Rehorn et al., 1996). While mutations in *pnr* and *grn* affect neither *lab* nor lab550 activity (data not shown), *srp* is essential for the development of the endoderm; in its absence, the endoderm does not form (Reuter, 1994) and it is therefore impossible to investigate the requirement of *srp* for *lab* or lab550 expression using loss-of-function alleles. With regard to the HMG proteins, these latter have been shown to act as architectural factors on enhancers (Grosschedl et al., 1994). Moreover, the SOX proteins, which also belong to the HMG family, can act as tissue-specificity factors (Kamachi et al., 2000; Pevny and Lovell-Badge, 1997). It is possible or even likely that one or several factors that bind to the 50 bp element recruits the Mad/Medea complex, resulting in its DPP sensitivity. This scenario would be similar to a number of situations reported for TGFβ or BMP signaling in vertebrates (Massague and Wotton, 2000).

As we have shown here, the HOMRE and the DPPRE show a strong interdependence (for a schematic representation of our results, see Fig. 10). None of the individual elements is capable of mimicking lab550 expression with regard to levels and spatial restriction; the expression driven by the HOMRE alone is much weaker, shifted posteriorly and does not respond to DPP; the activity of the DPPRE on its own is hardly detectable. It is only upon physical coupling of the HOMRE and the DPPRE that characteristic lab550 activity, i.e. strong DPP inducibility and HOX dependence, resumes. Therefore, we propose that functional interactions exist between the HOMRE and the DPPRE, and we identified a repressor element on the enhancer that might play an important role in these interactions.

The cooperation between the HOMRE and the DPPRE is controlled by a repressor

Why is the lab550 enhancer not activated by DPP in endodermal cells adjacent to ps 3, which also synthesizes the ligand? It has previously been argued that DPP is not taken up by anterior endodermal cells (Reuter et al., 1990). However, reporter constructs carrying oligomerized MAD-binding sites (5CRE; see Riese et al., 1997; Szuts et al., 1998) have been shown to respond strongly to DPP secreted by the anterior visceral mesoderm (as does lab550m300; this study). We found that a specific *cis*-acting element, site 300, is required to reduce dramatically the DPP response of the lab550 enhancer in the

entire endoderm; mutating site 300 in the DPPRE renders the latter highly responsive to DPP in all endodermal cells. From this finding, we conclude that a repressor, or a repressor complex, binds to site 300 on lab550 and tunes down the activity or the DPP inducibility of the DPPRE. The putative repressor is present in the entire *Drosophila* endoderm, but absent in the cultured cells used in this study; this conclusion is based on the observation that introducing the repressor site mutation in the cell culture reporter construct had only a minor effect on the DPP responsiveness of the enhancer in cell culture.

Based on our identification of this repressor element, we can envision several scenarios by which the functional cooperativity between the HOMRE and the DPPRE might be explained in molecular terms. Each model presents one extreme version of how regulation could in principle be achieved. It is obvious that these models represent oversimplifications, and that combinations of these and other scenarios will more accurately reflect regulation in vivo.

In a first scenario, the activators present on the HOMRE, added to those present on the DPPRE upon signaling, could lead to an overall positive output from labb550, overruling the activity of the repressor by the additivity of the activators binding to the two individual subelements upon induction. As the HOMRE is only occupied with the HOX protein complex in endodermal cells adjacent to ps 7, it is only in this region that the positive input would prevail.

In a second model, we propose that specific interactions between proteins binding on the HOMRE and the repressor(s) binding on the DPPRE might occur. These interactions could be direct, or could be mediated by a factor(s) that bridges the two enhancer elements. A potential interaction could involve inactivation of the repressor element via the HOX protein complex; this would allow the DPPRE to function only in the domain in which the HOMRE is occupied, therefore tightly linking the signaling response to segmental specificity. In favor of this hypothesis is our finding that the same elements, which drive the activated (derepressed) DPPRE (the MAD and the CRE sites as well as the 50 bp element), are also essential for the HOX-dependent activity of lab550.

In a third scenario, DPP signaling could have both a positive and a negative input on the DPPRE, and only in cells in which the linked HOMRE is occupied, the negative input could be counteracted (directly or indirectly). Some indications are in favor of this particular model (see below).

A crucial step towards the understanding of the molecular mechanisms that underlie the functional interaction between subelements of the lab550 enhancer will be to determine the nature of the repressor. Two nuclear proteins that can act as repressors in the DPP pathway have recently been described. Brinker (Brk) functions as a rather general and potent repressor of DPP targets and its expression is negatively controlled by DPP signaling itself (Campbell and Tomlinson, 1999; Jazwinska et al., 1999; Minami et al., 1999). BRK was recently shown to bind directly to several DPP target genes and inhibits their transcription (Rushlow et al., 2001; Sivasankaran et al., 2000; Zhang et al., 2001). Schnurri, a large nuclear zinc-finger protein, was recently shown to be required genetically for the DPP-dependent repression of *brk* transcription (Marty et al., 2000). It is intriguing that both BRK and SHN protein bind to the repressor site in vitro with high affinity (data not shown; see Grieder, 1997; Sivasankaran et al., 2000). However, lab550 expression is not affected in *brk* mutants, nor by single nucleotide mutations in site 300 that only affect Brk binding (data not shown). Nevertheless, we feel that it is possible that DPP signaling components play both a positive and a negative role in the regulation of lab550. Further experiments are required to investigate this possibility.

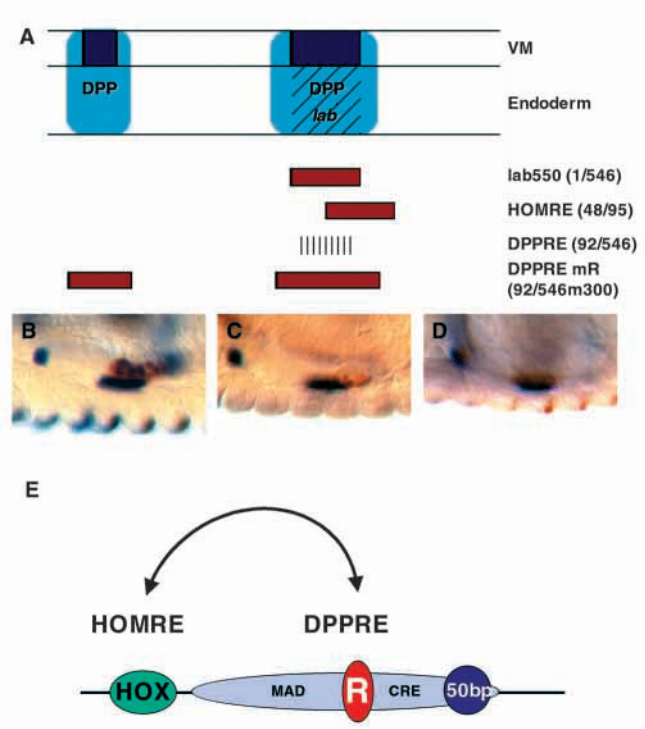

Fig. 10. The lab550 enhancer contains multiple elements that are all required for its HOX- and DPP-dependent regulation in the developing midgut endoderm. (A) Schematic representation of the developing midgut, depicting the visceral mesoderm (VM) and the endoderm. Cells in which DPP is transcribed are shown in dark blue; apparent distribution of the DPP ligand is indicated by the light blue squares. The expression of the endogenous *lab* gene in the endoderm is indicated by the stripes. The expression domains driven by lab550, HOMRE, DPPRE and DPPREm300 are shown below with brown bars. Embryos depicting the situation in stage 14 embryos are shown in B-D; DPP mRNA is in blue, and β-gal expression driven by lab550 (B), HOMRE (C) and DPPREm300 (D) in brown. A schematic summary of the factors that have been demonstrated or proposed to regulate lab550 expression is shown in E.

HOX proteins and the specificity of the response to DPP signaling

We have discussed how the requirement for a functional interaction between a DPPRE and HOMRE allows a HOX complex to control the cellular response to a signaling cascade, resulting in a segment-specific signal interpretation. The cooperative action of signaling mediators and tissue-specific factors on a single enhancer has been demonstrated previously (Flores et al., 2000; Halfon et al., 2000; Xu et al., 2000). However, the functional interaction of signaling mediators and a member of the HOX family of proteins on a single enhancer has not yet been reported in *Drosophila*. Therefore, lab550 could represent a paradigm for studying how signaling

mediators and tissue-specific factors are combined with HOX complexes to generate segmental differences along the anteroposterior axis of multicellular organisms.

Another example of such a segmentally regulated, signal-responsive element is the visceral mesoderm specific enhancer of the *dpp* gene, dpp674. This enhancer is directly regulated by Ultrabithorax (UBX), in conjunction with EXD (Capovilla et al., 1994; Chan et al., 1994); moreover, we found that dpp674 readily responded to ectopic DPP throughout the visceral mesoderm (data not shown). In its normal domain of activity in ps 7 of the visceral mesoderm, dpp674 could thus require both the activity of UBX/EXD and a direct input from the DPP pathway.

The embryonic enhancer of the *Distal-less* (*Dll*) gene could also be regulated by the combined activity of HOX proteins and signaling input, except that in this case, the action of HOX proteins would result in repression rather than activation. It has been demonstrated that the posterior HOX proteins UBX and ABD-A bind to two defined sites found at the 3′ end of the enhancer, thereby inhibiting its activity in the posterior segments of the ectoderm and restricting expression to the thoracic segments (Vachon et al., 1992). It was proposed (but not experimentally proven) that this enhancer is, in addition to this HOX control, under the direct control of the WG pathway, since expression of *Dll* in the leg primordia depends on *wg* (Vachon et al., 1992). Therefore, it is possible that the posterior HOX proteins directly block the activation of the Dll enhancer by WG signaling in the posterior segments and therefore impart segment specificity to the response to WG signaling.

There are also a large number of cases in which HOX proteins are required to regulate downstream genes in conjunction with signaling input, but in which detailed enhancer studies have not yet been done (Bilder et al., 1998; Henderson et al., 1999; Lints and Emmons, 1999; Maloof and Kenyon, 1998; Weatherbee et al., 1998).

In the light of these examples, we would like to propose that HOX proteins can determine the transcriptional response to signaling cascades in many different cellular systems by binding to common target enhancers. This way, HOX proteins could fulfill their recently proposed role as micromanagers (Akam, 1998), in combination with the activity of tissue-specific factors and nuclear effectors of signaling cascades, and lead to the segment-, tissue- and stage-specific activation of target genes, ultimately resulting in the progressive determination of different cell types. Our detailed analysis of the lab550 enhancer represents a first step towards the elucidation of the molecular mechanisms of HOX function with regard to signaling input in a developing organism. The systematic identification and analysis of regulatory elements carrying LAB/EXD/HTH sites in the *Drosophila* genome might provide a sufficiently large number of functional regulatory elements controlled by the same HOX protein to allow for more general conclusions concerning the molecular function of the products of the HOX genes and their link to cell signaling.

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