Tgfβ signaling acts on a Hox response element to confer specificity and diversity to Hox protein function

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Summary

Hox proteins play fundamental roles in generating pattern diversity during development and evolution, acting in broad domains but controlling localized cell diversification and pattern. Much remains to be learned about how Hox selector proteins generate cell-type diversity. In this study, regulatory specificity was investigated by dissecting the genetic and molecular requirements that allow the Hox protein Abdominal A to activate *wingless* in only a few cells of its broad expression domain in the *Drosophila* visceral mesoderm. We show that the Dpp/Tgfβ signal controls Abdominal A function, and that Hox protein and signal-activated regulators converge on a *wingless* enhancer. The signal, acting through Mad and Creb, provides spatial information that subdivides the domain of Abdominal A function through direct combinatorial action, conferring specificity and diversity upon Abdominal A activity.

Key words: Hox, Signaling, *Drosophila*, AbdA, Dpp/Tgfβ

Introduction

Patterning fields of cells is of central importance for the development of multicellular organisms. A few evolutionarily conserved molecules, including Hox selector genes and signaling molecules, play fundamental roles in these processes, which raises the question of how a restricted number of molecules instruct the cell fate complexity of developing animals. Hox selector genes encode homeodomain (HD)-containing transcription factors, whose functions distinguish the identities of homologous groups of cells along the anteroposterior axis (Lewis, 1978; McGinnis and Krumlauf, 1992). Previous experiments have established that Hox proteins (about 40 in vertebrates and only eight in *Drosophila*) play fundamental roles in coordinating the development of groups of cells during morphogenetic processes. A Hox combinatorial code has been proposed to instruct cellular and pattern diversity (Lewis, 1978; Hunt et al., 1991a; Hunt et al., 1991b), with cell fate determined by which combination of Hox transcription factors is active within it. Although the importance of the Hox code in patterning fields of cells is established, strong experimental support demonstrating that the combination of Hox proteins within a single nucleus instructs cell-type diversity is lacking.

Signaling and Hox protein functions have been extensively studied separately. However, how they act together to define higher levels of control is a poorly understood emerging theme. The *Drosophila* embryonic midgut provides an ideal model system for studying the coordinated action of Hox genes and signaling pathways. First, transcription of Hox genes in the visceral mesoderm (VM) occurs in adjacent non-overlapping expression domains (Tremml and Bienz, 1989), which allows a simple assessment of Hox protein function without any complication resulting from a potential Hox combinatorial code. Second, differential transcription of Hox genes directs localized production of two signaling molecules: Decapentaplegic/Tgfβ (Dpp/Tgfβ) in parasegment 7 (PS7) under Ultrabithorax (Ubx) control, and Wingless/Wnt (Wg/Wnt) in PS8 under Abdominal A (AbdA) control (Reuter et al., 1990; Bienz, 1994). The parasegmental boundary between PS7 and PS8 thus constitutes a signaling center from which the Dpp and Wg pathways organize morphogenetic processes: positioning the central midgut constriction (Staehling-Hampton and Hoffman, 1994) and establishing cell fate diversification (Hoppler and Bienz, 1994; Hoppler and Bienz, 1995). Third, the *Drosophila* midgut is the only tissue where multiple Hox target genes have been identified; these provide appropriate markers for investigating the mechanisms of Hox transcriptional activity at the molecular level (Graba et al., 1997).

We explored the genetic and molecular mechanisms that endow a single Hox protein with distinct transcriptional properties by studying the function of AbdA during midgut morphogenesis. AbdA is expressed and is active in the third and fourth compartments of the midgut (PS8-PS12), and yet it

activates the *wg* target gene only in PS8 (Immerglück et al., 1990). Here, we report that the Dpp signal secreted from PS7 provides the spatial information required for PS8-localized *wg* activation and that, acting through a newly identified 546 bp enhancer, AbdA and Mad, a transcriptional effector of the Dpp pathway, directly control *wg* transcription. The convergence of Hox function and Dpp signaling therefore occurs at the levels of DNA and transcription, and endows AbdA with PS8-specific regulatory properties.

Materials and methods

Identification, mutation of the XC enhancer and establishment of transgenic reporter lines

Restriction fragments from a 9 kb *wg* upstream regulatory region were cloned into pBS(SK+) (Stratagene) and then transferred to the P-element transformation vector pC4PLZ using standard cloning procedures. Mutated versions of the XC enhancer were generated using either the Sculptor mutagenesis kit (Pharmacia) or the Splicing by Overlap Extension (SOE) method (Horton et al., 1989). Details on the procedure and sequences of oligonucleotides used to generate XC variants are available upon request. The point mutations (underlined) introduced are as follows:

Hox/Pbx3, TGGATGGATGG→TGCTGCAGTGG;
Hox/Pbx1, AAGATCGAGTC→AAGAATTCGTT;
Hox6/7, GCAATTAGATTATG→GCAATCCGACTCTG;
DRS1, GCCGCCGCCG→GCTTCCTTCG;
DRS2, GGCGCCGCCTT→GGCACTTACTT;
DRS3, GCCGCCGA→TTTTCCGA;
Creb1, TGGCGCCA→TGGTTGAG; and
Creb2, TGACGCCG→TGATTGAT.

XC(Δ[Hox/Pbx2-3-4]) was generated by using an *Rsa*I restriction site to delete the promoter proximal region of the XC enhancer. Mutated enhancers and an oligomer containing three copies of Box2 were transferred into the pC4PLZ reporter vector, and introduced into the fly genome by P-mediated germline transformation (Rubin and Spradling, 1982). At least four lines were established and analyzed for each construct. In all experiments where *lacZ* expression levels were compared, embryos were processed in the same conditions and were stained for the same length of time.

wg midgut regulatory region from *D. virilis* and *D. pseudoobscura*

A *D. virilis* EMBL3 phage genomic library (provided by J. Tamkun) was screened with a 3.5 kb *Eco*RI/*Sph*I genomic fragment of the *D. melanogaster wg* upstream regulatory region. Hybridization was carried out, at moderate stringency, in 4×SSPE, 1% SDS, 0.5% non-fat dried milk. Washes were in 2×SSPE, 0.2% SDS, 0.05% sodium phosphate at the same temperature. From a phage clone containing a 7 kb *Sal*I fragment, a 1.4 kb *Bam*HI/*Hin*dIII restriction fragment that hybridizes to *D. melanogaster Xho*I/*Bam*HI DNA was subcloned in pUC19 and sequenced. The *D. virilis* sequence was PCR amplified, its sequence was verified, and it was then cloned into the pC4PLZ vector for P-mediated germline transformation. *D. pseudoobsura* sequences were from the *Drosophila* Genome Project.

Fly stocks and in situ hybridization

Fly stocks were obtained as follows: wg^{IL114} and wg^{CX4} from A. Martinez-Arias; dpp^{s4}, dpp^{s6} and dpp^{s13} from W. Gelbart; mad^{12} from S. Newfeld; *UAS-abdA* from M. Akam; *UAS-Creb(DN)*, also termed *UAS-Cbz*, *UAS-dpp* and *UAS-Tcf(DN*) from M. Bienz; hth^{P2} from R. Mann; *UAS-hth-en* from A. Salzberg; and *HS-abdA* from G. Morata. The exd^{XP11} allele and the *24B-Gal4* mesodermal driver were used. Mutant embryos were identified by the absence of *lacZ* balancers. In situ hybridization on wholemount embryos was performed as described by Tautz and Pfeifle (Tautz and Pfeifle, 1989), using antisense riboprobes produced by standard methods (Boehringer-Mannheim Genius kit). Immunostaining was performed according to Alexandre et al. (Alexandre et al., 1996), using the rabbit anti-β-galactosidase (Cappel). Embryos were mounted in 80% glycerol and photographed using Nomarski optics.

Protein production and gel shift assays

Full-length AbdA, Hth and Exd proteins for EMSAs were produced using the TNT-coupled in vitro transcription/translation system (Promega). The *Drosophila* CrebB (*CrebB-17A* – FlyBase) recombinant protein (Usui et al., 1993) was synthesized in *E. coli* and purified using Ni^{2+} chromatography (Qiagen). A GST-Mad fusion protein was produced and purified according to standard procedures (Pharmacia). It contained the first 159 amino acids of Mad, and thus included the MH1 DNA-binding domain (Waltzer and Bienz, 1999). The DIIRcon double-stranded oligonucleotides (Gebelein et al., 2002), and the following oligonucleotides and their respective complementary oligonucleotides, were used:

Box2, 5′-GGCCGCAATTAGATTATGCATTTTATGAAGTGCG-AATC-3′;
DRS1, 5′-CCAAAAATAGTCGGCGGCGGCTGAGTTTCT-3′;
DRS2, 5′-GCAGGTTTGGCGCCGCCTTGATGTCGCTGG-3′;
DRS3, 5′-TTTGACCCAATGCCGCCGACCCACGTAC-3′; and
Creb1.2, 5′-ATTCTTTTTGGCGCCAAGATCGAGTCGCGACC-TGGTGACGCCGTTGACGCTGCC-3′.

Box2m, DRS1m, DRS2m, DRS3m, Creb1-2m oligonucleotides and their complementary oligonucleotides are identical to the above oligonucleotides except that they carry the mutations indicated in the first section of Materials and methods. Oligonucleotides were end-labelled with [γ^{32}P]ATP, annealed with their respective complementary oligonucleotides, and gel purified. EMSAs with in vitro produced AbdA, Exd and Hth were performed in a volume of 20 μl as described by Pöpperl et al. (Pöpperl et al., 1995). Binding experiments were also performed with AbdA and Exd proteins produced in bacteria. In that case, His-tagged AbdA (from amino acid 79 to its carboxy terminus) and Exd (from amino acid 1 to 323) (Ryoo and Mann, 1999) were purified using Ni^{2+} chromatography (Qiagen). Binding experiments using Mad and *Drosophila* CrebB proteins were performed in similar conditions with 30,000 cpm radiolabelled probes. Binding buffers for Mad and *Drosophila* CrebB gel shifts were, respectively: 4% Ficoll, 20 mM Hepes (pH 7.9), 40 mM KCl, 1 mM EDTA and 4 mM DTT, with 2.5 μg BSA and 0.5 μg dAdT/10 μl of binding reaction; and 20 mM Hepes (pH 7.9), 20% glycerol, 100 mM KCl, 0.1% NP_4O, 20 mM $MgCl_2$ and 0.5 mM DTT, with 3 μg BSA/10 μl of binding reaction. DNA-protein complexes were analyzed by non-denaturing 6% PAGE in 0.5×TBE and were detected by autoradiography. The rabbit anti-AbdA antibody, raised against the full-length protein, was provided by M. Cappovila.

Results

Identification of a midgut enhancer that recapitulates *wg* expression and regulation

To identify the enhancer responsible for *wg* expression in the VM, subfragments of a 9kb genomic region known to drive *wg* embryonic expression (A. Martinez-Arias and L. Owen, personal communication) were analyzed in transgenic lines transformed with *lacZ* reporter constructs (Fig. 1A). The smallest fragment that drives accurate expression in the VM is a 546 bp *Xho*I/*Cla*I (XC) restriction fragment. Its activity is first detected during germ-band retraction (Fig. 1C), when *wg* transcripts are visualized in the VM by in situ hybridization (Fig. 1B), and only in PS8 VM cells. During subsequent development, XC enhancer activity still mimics *wg* expression (Fig. 1D,E), and is associated with the site of central midgut

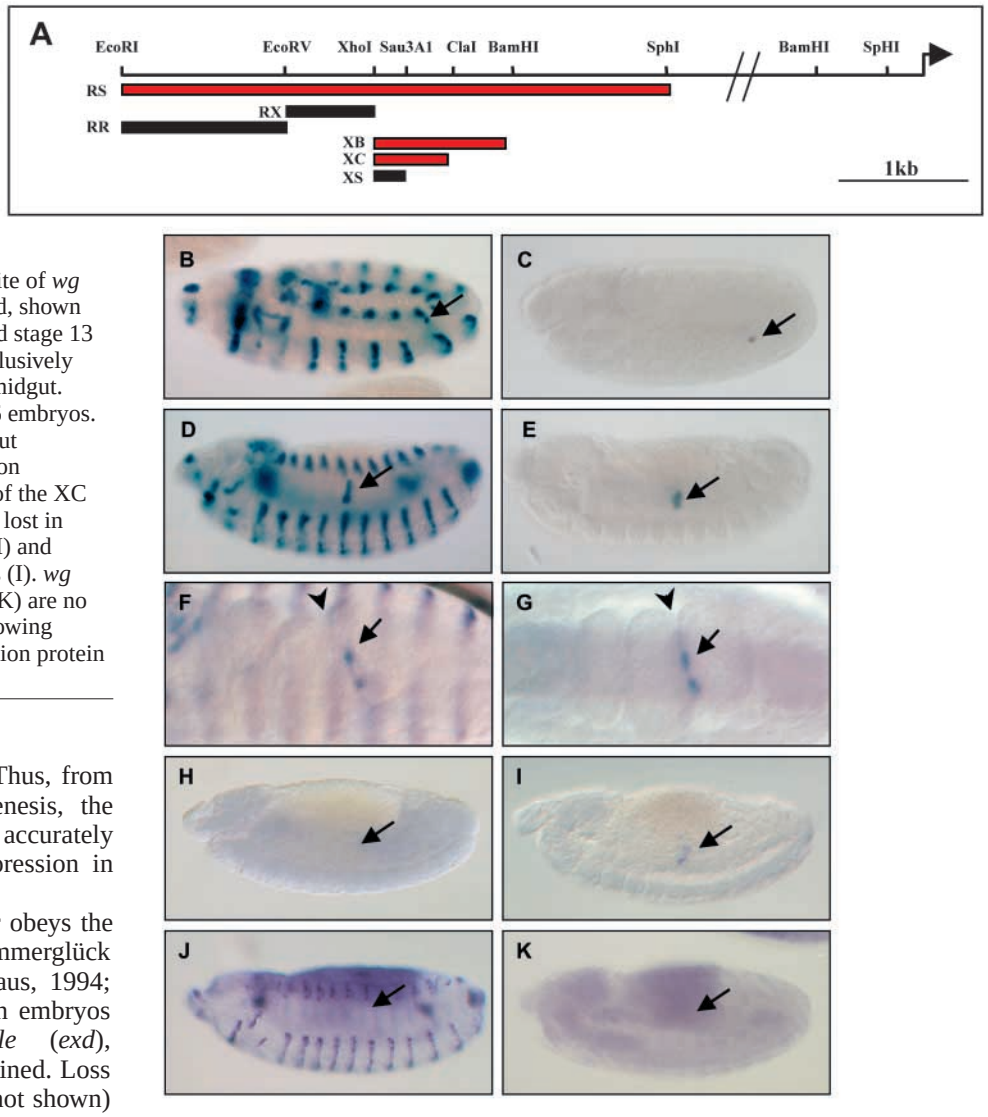

Fig. 1. Identification and regulation of a *wg* midgut enhancer. (A) *wg* upstream regulatory elements that drive (red bars) or do not drive (black bars) expression in the midgut when fused to a *lacZ* reporter. (B-G) Comparison of *wg* transcription and XC enhancer activity. *wg* trancripts (B,D,F) and *lacZ* (C,E,G) transcripts were detected by in situ hybridization. Arrows indicate PS8, the site of *wg* midgut expression. At all stages examined, shown here as lateral views of stage 11 (B,C) and stage 13 (D,E) embryos, XC enhancer activity exclusively mimics *wg* expression in the embryonic midgut. (F,G) Magnified ventral views of stage 16 embryos. The arrowheads indicate the central midgut constriction. (H-K) XC enhancer regulation recapitulates *wg* regulation. The activity of the XC enhancer visualized by *lacZ* transcripts is lost in $abdA^{JX2}$ homozygous-mutant embryos (H) and diminished in dpp^{s4} homozygous mutants (I). *wg* transcripts (J) and XC enhancer activity (K) are no longer detected in the central midgut following mesodermal expression of the Hth-En fusion protein in *24B-Gal4/UAS-hth-en* embryos.

constriction formation (Fig. 1F,G). Thus, from early on to the end of embryogenesis, the XC enhancer exclusively and accurately recapitulates *wg* spatiotemporal expression in the VM.

To establish that the XC enhancer obeys the same regulatory inputs as *wg* (Immerglück et al., 1990; Rauskolb and Wieschaus, 1994; Rieckhof et al., 1997), its activity in embryos mutants for *abdA*, *extradenticle* (*exd*), *homothorax* (*hth*) and *dpp* was examined. Loss of *abdA* (Fig. 1H), *exd* or *hth* (data not shown) function results in the absence of *lacZ* expression, indicating that the three transcription factors are essential for XC enhancer activation, as they are for *wg* transcription. In dpp^{s4} (Fig. 1I) or dpp^{s6} mutants (not shown), the activity of the XC enhancer is diminished, mimicing the decreased transcription of *wg* in these genotypes.

We analyzed in further detail the contribution of Hth to *wg* expression and XC enhancer control. Hth fulfils two separable functions in the regulation of Hox downstream target genes. It is responsible for Exd nuclear import (Rieckhof et al., 1997) and it can be a component of a tripartite Hox/Exd/Hth DNA-binding complex (Ryoo et al., 1999). To discriminate between these two functions, we used a fusion protein of Hth and the repression domain of Engrailed (En), which behaves as a dominant negative form of Hth but does not impair Exd nuclear translocation (Inbal et al., 2001). Expression of the Hth-En fusion protein in the mesoderm leads to the complete loss of *wg* transcription (Fig. 1J) and XC enhancer activity (Fig. 1K). This effect of Hth on *wg* is not a secondary consequence of a primary effect on *dpp*, as *dpp* expression in *hth* mutants is not abolished but is expanded anteriorly (data not shown), as it is in *exd*-mutant embryos (Rauskolb and Wieschaus, 1994). This suggests that Hth participates in a Hox/Exd DNA-binding complex that is required for *wg* control.

Dpp signaling is essential for *wg* expression and XC enhancer activity

dpp^{s4} and dpp^{s6} regulatory mutations do not completely abolish Dpp activity in the VM (Bilder et al., 1998): their effect on *odd paired* (*opa*) in the VM is weaker than is the effect of dpp^{s13}, a *shortvein* allele whose 3′ breakpoint is closer to the *dpp* transcription unit (Hursh et al., 1993). We found that *wg* transcription and XC enhancer activity are totally abolished in dpp^{s13} embryos (Fig. 2B,D). Dpp therefore is essential for *wg* transcription. A previous study reported that Dpp affects the level and maintenance of *wg* transcription (Immerglück et al., 1990), but we can see now, by using the stronger dpp^{s13} allele, that Dpp has an essential off/on influence. This is an important difference, as only an essential requirement for *dpp* is compatible with the Dpp signal providing the information responsible for PS8-restricted activation of *wg* by AbdA.

Dpp signaling provides positional cues for local *wg* expression and XC enhancer activity

To determine whether locally produced Dpp is responsible for the restricted *wg* activation by AbdA, we analyzed the changes in *wg* and XC enhancer expression patterns that result from expression of *abdA* and *dpp* at ectopic positions in the VM. Because the same conclusions were obtained for *wg* and the XC enhancer, we will describe the behavior of the enhancer only. We first provided the Dpp signal ubiquitously in the VM and observed additional patches of β-galactosidase staining (Fig. 2E). The sites of ectopic expression are posterior to PS8 within the AbdA expression domain. Most embryos exhibit two additional patches, whereas in a few cases a third patch is observed more posteriorly. This suggests that XC enhancer activation requires a high level of Dpp signaling, which is best achieved close to endogenous sources of Dpp, where endogenous and 24B-driven Dpp signal are combined. At later stages, ectopic Dpp and, consequently, ectopic *wg* expression, here visualized by posterior ectopic XC enhancer activity (Fig. 2F), results in abnormal midgut morphogenesis, with ectopic constrictions forming just posterior to the central one.

We next analyzed XC activation in response to ubiquitous expression of AbdA in the mesoderm and could occasionally detect a faint ectopic β-galactosidase staining anterior to the normal site of *wg* expression, close to PS7 (Fig. 2G). This experiment deserves two comments. First, the low frequency and reduced levels at which ectopic staining occurs is a consequence of two opposite functions of AbdA in the VM. Besides activating *wg*, AbdA represses *dpp* (Reuter et al., 1990), which indirectly impairs *wg* transcription. Thus, the embryos in which ectopic *lacZ* expression is seen likely correspond to embryos where AbdA has not completely abolished *dpp* transcription. Second, the fact that ectopic staining is only seen close to PS7, where the Dpp signal originates, is further consistent with the requirement of both AbdA and Dpp for XC enhancer activation.

Table 1. In vivo activities of XC variants

XC enhancer variants	PS8 VM activity	Figures
XC	+++	1C,E,F
XC(Hox/Pbx1)	++	NS
XC(Hox/Pbx2)	++	NS
XC(Δ[Hox/Pbx2-3-4])	++++	4D
XC(Hox/Pbx1;Δ[Hox/Pbx 2-3-4])	++++	NS
XC(ΔBox2)	+	4B
XC(Hox6/7)	+	4C
XC(Hox6/7;Δ[Hox/Pbx2-3-4])	+	4E
XC(DRS1-3)	+++	NS
XC(DRS-2-3)	–	5D
XC(Creb1-2)	+	5E
XC(DRS1-2-3;Creb1-2)	–	5F

NS, not shown.

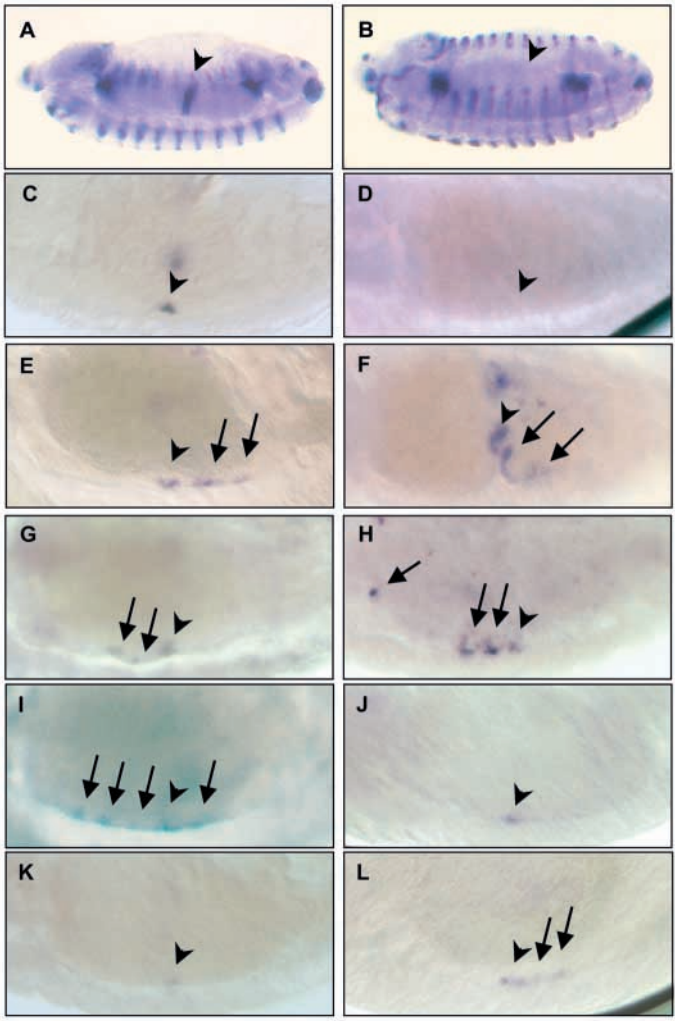

Fig. 2. *wg* expression and XC enhancer activity depends on Dpp and Wg signaling. (A,B) *wg* transcripts revealed by in situ hybridization. Arrowheads indicate PS8, the site of *wg* midgut expression in wild-type embryos (A). *wg* expression is completely abolished in the midgut of dpp^{s13} homozygous-mutant embryos (B). (C-L) Regulation of XC or XC(Δ[Hox/Pbx2-3-4]) enhancer activity by AbdA, Dpp and Wg visualized by in situ hybridization to *lacZ* transcripts. All panels show magnifications of lateral views of the midgut of stage 14 embryos, except panel F, which is from a stage 15 embryo. Embryos in C,J,K and L have been processed in the same conditions and the length of staining time was identical. Arrows indicate sites of ectopic enhancer activity. The embryo in C bears a wild-type copy of the XC enhancer and serves as a reference for the activity of XC variants. With respect to *wg* expression, XC enhancer activity in the wild type (C) is lost in dpp^{s13} homozygous-mutant embryos (D). The embryo in D has been overstained to ensure the absence of *lacZ* staining. Mesodermal ubiquitous expression of Dpp in *24B-Gal4/UAS-dpp* embryos induces ectopic activity of the XC enhancer posterior to PS8 (E) in cells where AbdA is present. At stage 15, ectopic sites of XC activity coincide with the sites of extra constrictions that form in this genotype (F). Mesodermal ubiquitous expression of AbdA in *24B-Gal4/UAS-abdA* embryos weakly induces ectopic activity of the XC enhancer anterior to PS8 (G), in close proximity to the PS7 Dpp source. In *24B-Gal4/UAS-abdA* embryos, ectopic XC(Δ[Hox/Pbx2-3-4]) enhancer activity is stronger than XC enhancer activity, and is also detected more anteriorly, close the source of Dpp in VM cells close to PS3-4 (H). Simultaneous expression of AbdA and Dpp in *24B-Gal4/UAS-dpp/UAS-abdA* embryos induces ectopic XC enhancer activity anterior and posterior to PS8 (I). XC activity is diminished in wg^{IL114} homozygous embryos shifted to restrictive temperature at 7 hours of development at 25°C (J) or in *24B-Gal4/UAS-Tcf(DN)* embryos expressing the dominant-negative form of the Wg transcriptional effector *Drosophila* Tcf (K). In wg^{ILL114} homozygous mutants grown at 29°C, ectopic Dpp signaling provided by *24B-Gal4/UAS-dpp* still induces, although at lower levels, ectopic XC enhancer activity (L).

However, we never detected XC enhancer activity close to PS3-4 of the VM, where Dpp is also produced. To examine this point further, we used a XC enhancer version lacking the most proximal sequence, XC(Δ[Hox/Pbx2-3-4]), which has stronger activity than does the full-length enhancer (see Table 1 and Fig. 4D). We first checked that ectopic Dpp, as with the XC enhancer, induces posterior ectopic XC(Δ[Hox/Pbx2-3-4]) activity (not shown). The improved activity of this enhancer allowed a better visualization of the effect of ubiquitously provided AbdA (Fig. 2H): as for XC, two sites anterior to the normal site of *wg* expression were observed. In addition, ectopic staining then also occurred more anteriorly, at the site of Dpp production in PS3-4. These experiments clearly emphasize the simultaneous requirement of Dpp and AbdA for XC enhancer and *wg* transcriptional activation. Thus, the local source of Dpp, secreted from cells of PS7, just anterior to the large AbdA expression domain (PS8-12), allows PS8-restricted activation of *wg* by AbdA.

To determine whether all VM cells are competent to express *wg* in response to AbdA and Dpp, we looked at XC and XC(Δ[Hox/Pbx2-3-4]) enhancer activity following simultaneous expression of AbdA and Dpp in the entire VM. In this context, ectopic *lacZ* expression occurs both anterior and posterior to PS8 (Fig. 2I). However, we did not observe ectopic expression in all VM cells. Thus, although many VM cells are competent to activate the enhancers when exposed to Dpp and AbdA, some cells do not respond in our experimental conditions. We suggest that the threshold of 24B-driven Dpp is limiting. This is supported by the two following observations. First, when providing 24B-driven Dpp, *wg* expression or XC enhancer activity always occurs close to the source of endogenous Dpp, but rarely at positions where the ubiquitous source of Dpp is not implemented by the endogenous signal. Second, when strong *dpp* expression is induced in the anterior midgut by the ectopic expression of an AbdA protein mutated in the hexapeptide motif, strong and ubiquitous expression of *wg* in all VM cells of the anterior midgut is achieved (Merabet et al., 2003).

Wg signaling implements the AbdA and Dpp responsiveness of the XC enhancer

As Dpp and Wg act together in the regulation of a Ubx VM enhancer (Eresh et al., 1997; Riese et al., 1997), we examined whether XC activity in PS8 depends on Wg signaling. XC activity is severely reduced in the absence of *wg* function (Fig. 2J), or in the presence of a dominant-negative form of *Drosophila* Tcf (Pan – FlyBase), a transcriptional effector of Wg signaling (Brunner et al., 1997) (Fig. 2K). Consistent with its dependency on Wg signaling, ectopic activation of the XC enhancer by ubiquitous *dpp* expression in the VM occurs at

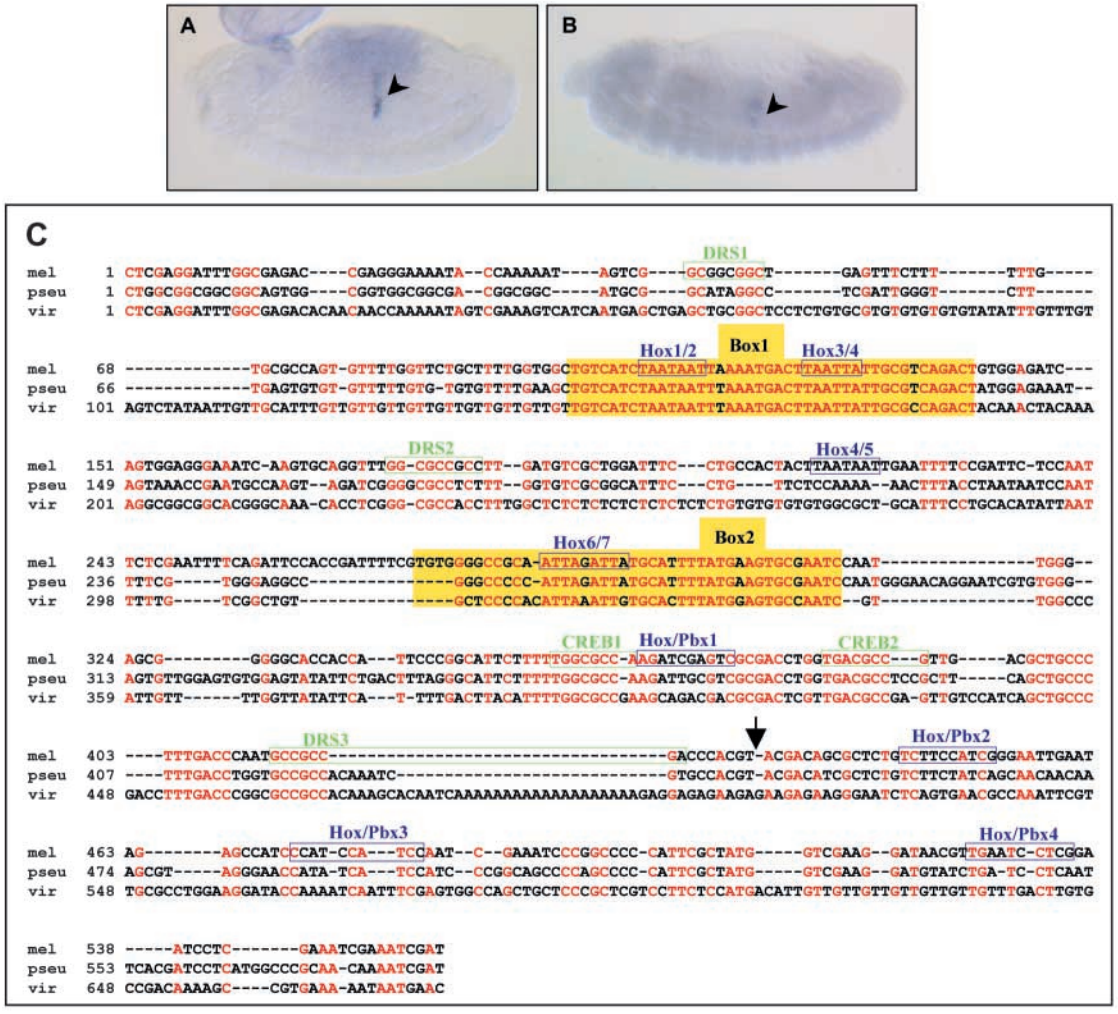

Fig. 3. Evolutionary conservation of the *wg* midgut enhancer. (A,B) Enhancer activities visualized by in situ hybridization to *lacZ* transcripts. Embryos are shown in a lateral view. The *D. virilis* counterpart of the XC enhancer drives expression in the central part of the midgut (B), as does the *D. melanogaster* XC enhancer (A), although at a reduced level. (C) Alignment of the *D. melanogaster* (mel), *D. pseudoobscura* (pseu) and *D. virilis* (vir) sequences. Sequences in red emphasize sequence identity. Two long stretches of sequence conservation are boxed in yellow. Consensus binding sites are indicated in blue for Hox and Hox/Pbx complexes, and in green for Mad/Medea (referred to as DRS) and Creb proteins. Sequences after the arrow are deleted in XC(Δ[Hox/Pbx2-3-4]).

high levels only when *wg* is also present (compare Fig. 2L with Fig. 2E). In summary, these observations show that both Dpp and Wg control *wg* transcription, each providing a distinct contribution: Dpp is essential and instructive, allowing local activation of *wg* by AbdA, whereas Wg is permissive, necessary for XC enhancer activity but not controlling spatial pattern. The conclusion reached here, from loss-of-function experiments, that Wg maintains its own expression through an auto-regulatory loop, is distinct from the conclusion obtained by others, from gain-of-function experiments (Yu et al., 1998), that high level Wg signaling represses its own expression.

Potential binding sites for AbdA and transcriptional effectors of the Dpp signaling pathway are evolutionarily conserved in the XC enhancer

To address whether AbdA and Dpp signaling could directly regulate *wg*, we first examined the sequence of the XC enhancer for the presence of putative binding sites for AbdA and for Mad/Medea (referred to as DRS, for Dpp response sequence), the canonical transcriptional effectors of the Dpp/Tgfβ signaling pathway known to recognize identical target sequences (Affolter et al., 2001). As genetic and molecular data led to the proposal that, in *Drosophila*, the CRE sequences to which Creb proteins bind are required to respond to Dpp in addition to DRSs (Andrew et al., 1997; Eresh et al., 1997), we also looked for potential Creb binding sites. Six TAAT core sequences and four sequences resembling the consensual Hox/Pbx binding sites (TGATNNATG/TG/A) were identified as potentially mediating AbdA function (Fig. 3C). The Hox/Pbx 3 and 2 sequences strongly match the consensus, with seven or six of the eight consensus nucleotides conserved, respectively. Hox/Pbx sequences 1 and 4 only have five of the eight consensus nucleotides conserved. The XC fragment contains three sequences matching DRSs and two potential CRE sites.

To assess the evolutionary conservation of the XC enhancer, an homologous fragment from *Drosophila virilis* was isolated and analyzed for its in vivo activity by transgenesis in *Drosophila melanogaster*. The *D. virilis* fragment drives expression in a pattern very similar to that of the XC enhancer (Fig. 3B), suggesting that sequences conserved between these two enhancers may be important for *wg* regulation in the midgut. Sequence comparison, including sequences from *D. pseudoobscura*, revealed that a majority of the TAAT core motifs, the DRSs and the putative Creb-binding sequences are evolutionarily conserved, whereas sequences that match heterodimeric Hox/Pbx consensus binding sites are not (Fig. 3C). We also noted the existence of two large conserved sequences, Box 1 and 2. As Box1 lies in a fragment that does not drive reporter gene expression in transgenic flies (XS in Fig. 1A), particular attention was paid to Box2 (see below).

AbdA directly regulates *wg* and mediates its effect through multiple binding sites

To test whether *wg* is a direct target of AbdA, and to identify the cis-regulatory sequences responsible for this regulation, we generated variants of the XC enhancer disrupted in one or several of the potential Hox-binding sites and analyzed their activities in vivo. We first looked at Hox6/7 motifs found in the evolutionarily conserved Box2 and obtained evidence that they are important for the *wg* response to AbdA. A variant deleted of Box2 showed a severely reduced in vivo activity (Fig. 4B).

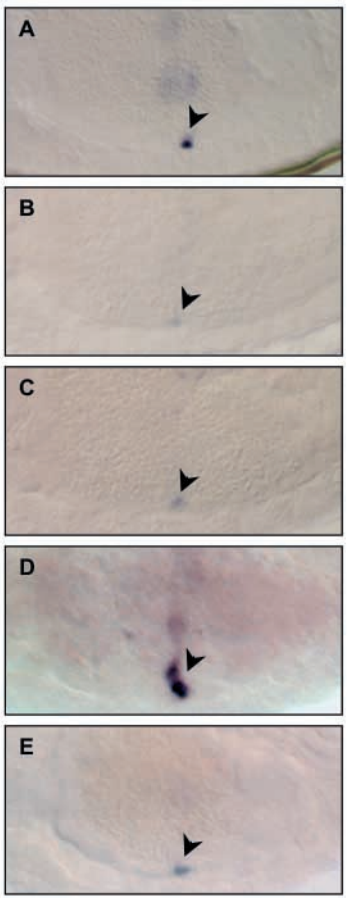

Fig. 4. Two Hox binding sites within Box2 are required for XC enhancer activity. Functional requirement of Hox6/7 sites for XC or XC(Δ[Hox/Pbx2-3-4]) enhancer activity visualized by in situ hybridization to *lacZ* transcripts. All embryos have been processed under the same conditions, with identical staining times. All panels show magnifications of lateral views of the midgut of stage 14 embryos. The embryo in A bears a wild-type copy of the XC enhancer and serves as a reference for the activity of XC variants. The deletion of Box2 (B), or the mutation of the two Hox binding sites (Hox6/7) found in Box2 (C), results in a strong diminution of XC enhancer activity. The activity of the XC(Δ[Hox/Pbx2-3-4]) enhancer (D) is stronger than that of the full-length XC (A), and is also significantly diminished upon mutation of the Hox6/7 sites (E).

A similar loss of enhancer activity was obtained by mutating the two Hox TAAT core motifs (Fig. 4C), suggesting that the diminished activity observed following the deletion of Box2 results from impairing the AbdA-regulatory function.

Because the deletion of Box2 does not cause a complete loss of *lacZ* gene expression, as was observed upon *abdA* mutation, we investigated whether the four putative sites for Hox/Pbx lying outside of Box2 play a role in AbdA-mediated activation of the XC enhancer. Enhancer variants were generated and tested in transgenic flies. Point mutations that alter Hox/Pbx site 1, which lies between two Creb-binding sites, or Hox/Pbx site 3, which closely matches the Hox/Pbx consensus, lead only to a weak inactivation of the XC enhancer (data not shown; summarized in Table 1). More drastically mutated variants, XC(Δ[Hox/Pbx2-3-4]), where the promoter-proximal region containing Hox/Pbx sites 2, 3 and 4 is deleted, and XC(Hox/Pbx1;Δ[Hox/Pbx2-3-4]), which no longer contains any potential Hox/Pbx binding sites, do not reduce enhancer activity but, surprisingly, improve it (Fig. 4D and Table 1, respectively). This suggests that the deleted region contains sites used to downregulate the XC enhancer. In summary, these data show that AbdA directly regulates *wg*, and that it does so through multiple binding sites.

To establish more firmly the importance of Box2 in mediating the response to AbdA, two additional experiments were performed. First, we used the XC(Δ[Hox/Pbx2-3-4]) that displays a stronger enhancer activity than the full-length enhancer version, and found that the two TAAT core sequences

of Box2 play an essential role, as their mutation results in decreased enhancer activity (Fig. 4E). Second, we assayed the ability of Box2 to drive, on its own, reporter gene expression in transgenic flies. Box2 initially promotes expression in a group of cells within the prospective third midgut chamber (Fig. 5A), posterior to *wg*-expressing cells. Later in development (stage 15), enhancer activity is detected in the entire third midgut chamber and part of the fourth gut chamber (Fig. 5B). Box2 thus promotes expression in a posteriorly extended domain with regards to the *wg*/XC domain. However, it is limited to VM cells that express AbdA, suggesting a strict dependence on AbdA. The lack of any β-galactosidase staining in *abdA* mutants (Fig. 5C), and the induction of *lacZ* expression in the whole VM of embryos producing AbdA throughout this germ layer (Fig. 5D), clearly demonstrates that Box2 activity is controlled by AbdA.

As Box2 is sufficient to generate an AbdA-dependent expression pattern and crucially contributes to XC enhancer activity, we assayed for in vitro molecular interactions. Band-shift experiments established that in vitro produced AbdA protein specifically binds to Box2. This binding (Fig. 5E; lane 6) depends on the integrity of the two TAAT core sequences (Fig. 5E; lane 11) and is abolished when anti-AbdA antibodies, which impair AbdA DNA binding (Fig. 5E; lane 23), are added to the binding reaction (Fig. 5E; lane 10). Together with the in vivo activity of Box2, these results indicate that AbdA binding to Box2 directly regulates *wg* expression.

Although Box2 does not contain any consensus sequences for Hox/Pbx, EMSA experiments in the presence of Exd were conducted. AbdA and Exd produced in vitro do not form a dimeric complex on Box2 (Fig. 5E; lane 7), contrasting with the ability of the two proteins (same batches) to assemble on DllRcon, an enhancer element of *Distalless* (Gebelein et al., 2002) that recruits an AbdA/Exd complex (Fig. 5E; lane 21) (Merabet et al., 2003). EMSA performed using AbdA (from amino acid 79 to the carboxy terminus) and Exd (from amino acid 1 to 323) variant proteins produced in *E. coli* led to the same conclusion: that AbdA and Exd do not form a dimeric complex on Box2 (data not shown). During these experiments, we noticed that proteins produced in vitro and in *E. coli* behaved differently with respect to the effect of Exd on the DNA-binding activity of AbdA: whereas DNA-binding was slightly decreased using in vitro produced proteins (Fig. 5E; lane7), it was significantly improved using proteins produced in *E. coli* (not shown). This suggests either that the folding of the in vitro and bacterially produced proteins are not equivalent, or that domains absent from the proteins produced in *E. coli* inhibit the improvement of AbdA DNA binding by Exd. A similar improvement of Hox DNA binding activity by Exd in the absence of Hox/Exd complex formation (Pinsonneault et al., 1997; Ryoo and Mann, 1999; White et al., 2000) has already been reported, suggesting that Exd/Pbx cofactors use multiple molecular mechanisms for assisting Hox protein function.

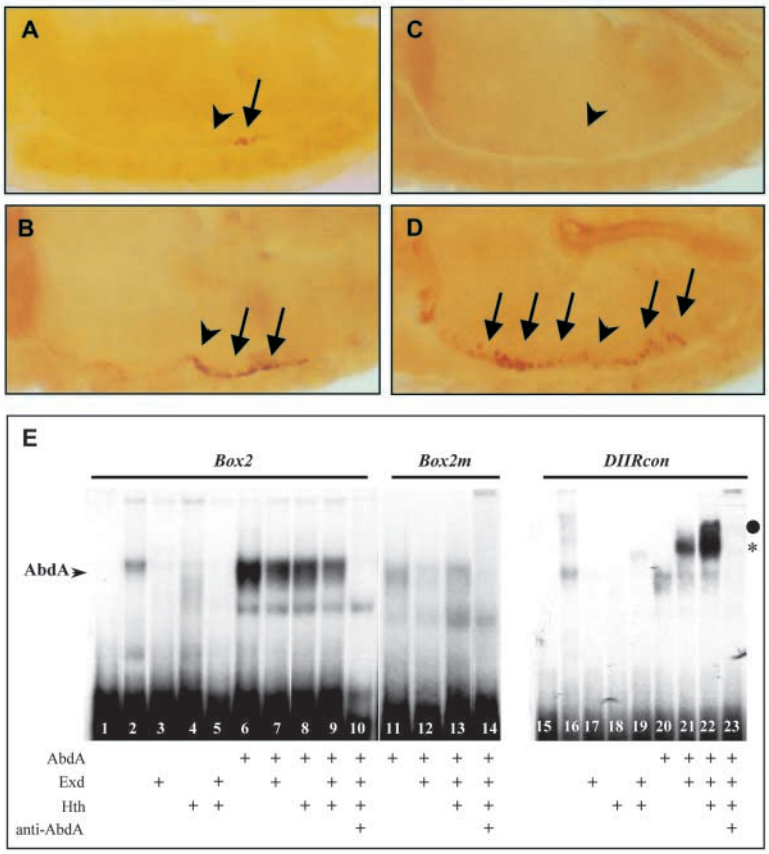

Fig. 5. Box2 binds in vitro to AbdA and is sufficient to drive an AbdA-dependent expression pattern in the embryonic midgut. (A-D) Box2 responds in vivo to AbdA. Enhancer activity is visualized by immunohistochemistry using an anti-β-galactosidase antibody. All panels show magnifications of lateral views of the midgut of stage 14 embryos. Arrowheads indicate PS8, the site of *wg* midgut expression. Arrows indicate ectopic enhancer activity. An oligomer consisting of three copies of Box2 drives *lacZ* expression in the posterior midgut in the AbdA expression domain. Expression is first detected posterior to the normal site of *wg* expression (A) and later in a domain that includes the third, and part of the fourth, midgut chambers (B). Box2 enhancer activity no longer occurs in *abdA*^{JX2} homozygous mutants (C), and is induced ectopically in the entire midgut VM when AbdA is ubiquitously provided by a heat shock construct (D). (E) Gelshift experiments with AbdA, Exd and Hth proteins produced in vitro were performed on wild-type (lanes 1-10) and mutated (Box2m; lanes 11-14) forms of Box2. Box2m carries the same point mutations as those introduced in XC(Hox6/7). 3 μl of the programmed lysate were used for each protein and for the mock lysate (lanes 2 and 16). For the binding experiments combining Exd and Hth, the two proteins were simultaneously produced and 6 μl of the lysates were used. The anti-AbdA serum was used at a 1/20 dilution. The activity of the proteins were assayed on a DllR sequence (lanes 15-23), known to assemble an AbdA/Exd/Hth complex. The asterisk and the dot mark the position of the AbdA/Exd and AbdA/Exd/Hth complexes, respectively.

In addition, we asked whether the presence of Hth allowed the formation of an AbdA/Exd/Hth complex on Box2. Consistent with the absence of a sequence matching a Hth binding site, no AbdA/Exd/Hth complex was observed on Box2 (Fig. 5E; lane 9), although the same preparations of proteins do form a tripartite complex on DllRcon (Fig. 5E; lane

22). In summary, these observations do not favor a model whereby AbdA, Exd and Hth act as a ternary protein complex binding Box2 in the regulation of *wg*, as has been demonstrated in the regulation of *labial* (Mann and Affolter, 1998). However, they do not exclude that aided by additional proteins and cis-regulatory sequences, such a ternary complex may form in vivo.

The Dpp transcriptional effector Mad and the *Drosophila* CrebB protein directly regulate *wg*

First, we addressed whether Mad and Creb are involved in XC enhancer activation. In embryos transformed with the XC-*lacZ* construct and mutant for *mad*, no β-galactosidase staining could be detected (Fig. 6B), indicating that Mad is essential for XC enhancer activity. As no mutant for *Drosophila CrebB*, the gene encoding the Creb isoform expressed in the VM, is available, we used a dominant-negative form of Creb. Its expression in the mesoderm strongly reduces β-galactosidase staining (Fig. 6C), indicating that a Creb protein, most likely *Drosophila* CrebB, is required for XC enhancer activity.

Next, we determined whether the evolutionarily conserved consensus sequences for Mad/Medea (DRS1, 2, 3) and Creb are used in vivo. The mutation of DRS 1 and 3 does not result in a significant inhibition of the reporter gene (data not shown; Table 1). When all three DRSs are simultaneously mutated, the XC enhancer is inactive in the VM (Fig. 6D), clearly demonstrating the essential role of Mad/Medea consensus sequences for *wg* VM expression. This result indicates, in addition, that the three DRSs individually contribute to the control by Dpp transcriptional effectors, or ,alternatively, that DRS2 is of special functional importance. Of note, the variant mutated for the three DRSs gains a novel activity, as revealed by ectopic *lacZ* expression near the foregut/midgut boundary and in the midgut endoderm close to sources of Dpp signal. Interaction of Mad/Medea with the DRSs therefore appears to be distinctly used in PS8 of the VM for *wg* activation, in the midgut endoderm and more anteriorly to prevent *wg* expression. These observations suggest that the function ultimately depends on locally specified, tissue-specific, combinatorial interactions. Mutation of the two Creb-binding sites reduces XC enhancer activity, indicating that, although important, they are not essential (Fig. 6E). The complete loss of XC enhancer activity observed when the three DRSs and the two Creb consensus sequences are mutated (Fig. 6F) indicates that the ectopic endoderm expression seen with XC(DRS1-2-3) requires Creb binding.

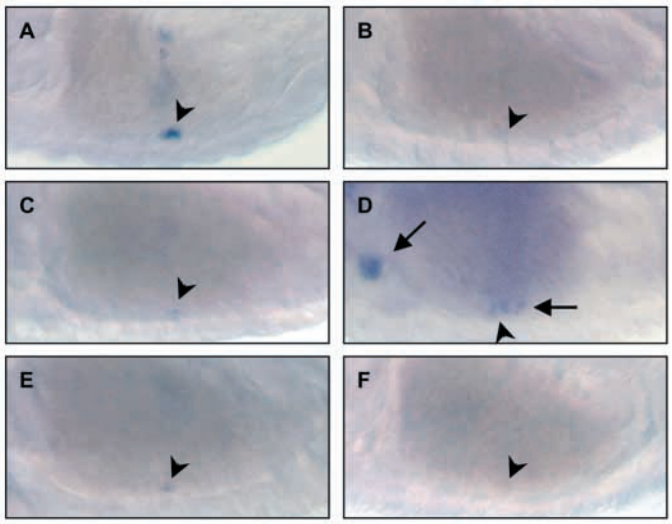

Fig. 6. Requirement of Mad and Creb proteins for XC enhancer activity. Enhancer activity is visualized by in situ hybridization to *lacZ* transcripts. All panels show magnifications of lateral views of the midgut of stage 14 embryos. Arrowheads indicate the site of *wg* expression in PS8. Arrows indicate ectopic enhancer activity. All embryos have been processed under the same conditions and the staining times were identical. (A-C) Requirements in trans. (A) Wild-type embryo carrying the XC enhancer. The activity of the XC enhancer is lost in mad^{12}-homozygous mutants (B) and is strongly reduced upon expression of a dominant-negative form of *Drosophila* CrebB in the mesoderm of *24B-Gal4/UAS-Creb(DN)* embryos (C). (D-F) Requirements in cis. Mutation of the three DRS in XC(DRS1-2-3) results in the complete loss of enhancer activity in VM PS8 (D). It also induces ectopic activity (arrows) of the enhancer in endodermal cells from the central part of the midgut, and in a more anterior region close to the foregut/midgut boundary (D). XC(Creb1-2) that bears mutations in the two Creb binding sites shows a severely reduced enhancer activity (E). The mutation of the three DRSs and the two Creb binding sites results in a complete loss of enhancer activity (F), including in the territories where the enhancer is ectopically induced by XC(DRS1-2-3).

In addition, we tested whether Mad and *Drosophila* CrebB proteins directly bind their putative sites on the XC enhancer in vitro. Band-shift experiments performed with purified proteins show that DRS1, 2 and 3 bind to Mad with distinct affinities (Fig. 7A-B; data for DRS3 not shown). The strongest binding is to DRS2, which might be functionally significant as XC(DRS1-3), a variant mutated in sites 1 and 3 only, possesses an in vivo activity comparable to the wild-type version. The in vitro association of Mad to each of the three sequences appears specific, as shown by the impaired binding when each DRS is mutated, as well as by the competition experiments. Similar band-shift experiments conducted with *Drosophila* CrebB purified proteins also led to the conclusion that *Drosophila* CrebB specifically binds to Creb1 and 2 consensus sequences (Fig. 7C). In vertebrates, Smads and the Creb-like proteins Fos and Jun have been shown to co-activate artificial promoters (Zhang et al., 1998). It therefore appears that Creb proteins may play a rather general role in implementing the response to Dpp and possibly other Tgfβ signaling molecules.

In summary, these experiments show that Mad, and most likely Medea that is known to function in a complex with Mad, as well as Creb proteins bind in vitro sites that are specifically required for the activation of the *wg* XC enhancer in vivo. This provides strong evidence that the Dpp signaling pathway directly regulates *wg*.

Discussion

Hox/signaling integration: interactions for reciprocal profits

Considerable interest has recently emerged about how selector gene products and signaling molecules cooperate in organ patterning (Curtiss et al., 2002). It was proposed that combinatorial use of Scalloped (Sd), a transcription factor that works together with Vestigial (Vg) to specify the wing field (Bray, 1999), and transcriptional effectors of the Notch [Suppressor of Hairless, Su(H)] (Lecourtois et al., 1995) and

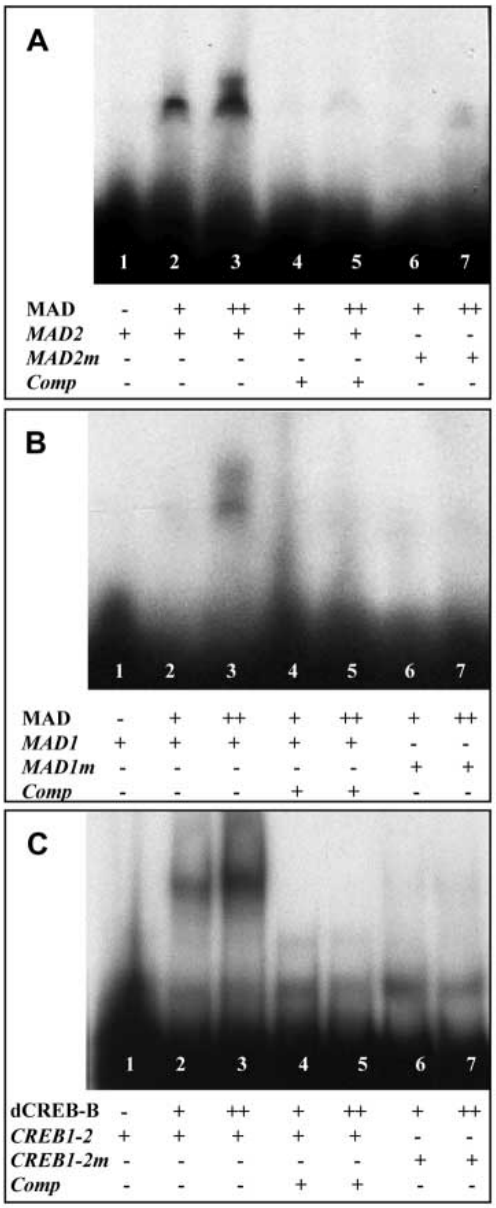

Fig. 7. Mad and *Drosophila* CrebB proteins bind in vitro to the XC enhancer. (A) Gelshift experiments with Mad protein [50 ng (+) or 200 ng (++)] were performed on double-stranded oligonucleotides corresponding to wild-type or mutated (DRS2m) versions of DRS2, in the presence or absence of a 500-fold excess of cold DRS2 competitor. Lanes 1-3 show a dose-dependent binding of Mad to DRS2. Lanes 4-7 indicate that binding is competed by cold DRS2 (lanes 4 and 5), and that the integrity of the DRS2 site is required for Mad binding (lanes 6-7). (B) Similar experiments on double-stranded oligonucleotides corresponding to the wild-type or mutated (DRS1m) version of DRS1. Compared with the experiments in A, this gelshift shows that Mad protein binds DRS2 with a stronger affinity than DRS1. (C) Gelshift experiments with *Drosophila* CrebB protein [20 ng (+) or 100 ng (++)] were performed on double-stranded oligonucleotides corresponding to wild-type (Creb1-2) or mutated (Creb1-2m) versions of Creb-binding sites, in the presence or absence of a 500-fold excess of cold Creb1-2 competitor. Lanes 1-3 show a dose-dependent binding of *Drosophila* CrebB. Lanes 4-7 indicate that the binding of *Drosophila* CrebB is competed by the competitor, and that the integrity of the two Creb binding sites is required for binding to occur.

Dpp (Mad) signaling pathways regulate *cut* (*ct*) and the *vestigial* quadrant enhancers (*vgQ*) in specific portions of the wing disc (Guss et al., 2001). *vgQ* and *ct* are direct targets of Sd, and the association of binding sites for Sd to those of Mad or Su(H), creates synthetic enhancers that mimic *vgQ* or *ct* expression. The absolute requirement for Sd-binding sites in the synthetic enhancers provided an explanation for the activation of *ct* and *vgQ* by the Notch and Dpp pathways in the wing disc only. Two additional studies showed that the tissue specific transcription factors Twist and Tinman also locally specify the activity of signaling pathways (Halfon et al., 2000; Marty et al., 2001; Xu et al., 1998). Thus, selector proteins provide tissue-specificity for the action of signaling molecules, allowing a few signals to be reiteratively used and yet achieve distinct functions in different tissues. This conclusion also holds for the Hox selector protein Lab, which is involved in a positive autoregulatory loop in the endoderm. Although Dpp signals in the central midgut both in the VM and in the endoderm, *lab* expression and the activity of a *lab* Dpp-responsive enhancer only occurs in the endoderm (Grieder et al., 1997). It was further shown that the enhancer contains a single Lab/Exd/Hth composite binding site responsible for the endoderm-restricted activity (Marty et al., 2001).

Like signaling molecules, Hox proteins are also widely expressed and reiteratively used during development. Although the Lab/Dpp synergy provides the best documented example of Hox/signaling combined action, it does not constitute a suitable model to address whether signaling pathways modulate and specify Hox protein activity, because synergy between Lab and Dpp apparently occurs in all Lab-expressing cells. In this study, Hox/signaling integration was examined to determine whether signaling pathways contribute towards specifying how a widely expressed Hox selector protein controls the development of distinct pattern elements at different locations. We show that the Dpp signal secreted from PS7 provides the positional cue responsible for localized activation of *wg* by AbdA. Biochemical and reverse genetics experiments established that AbdA and Mad directly regulate *wg* transcription through the XC enhancer, which thus serves as an integrator of Hox and Tgfβ input. AbdA is impotent with respect to this enhancer in the absence of the Dpp signal, though it can function perfectly well on other genes without Dpp (Bilder et al., 1998). Therefore, functional interactions between selector proteins and signaling pathways confer specificity to signaling pathways (Curtiss et al., 2002; Guss et al., 2001), and reciprocally confer functional diversity to selector proteins (this study).

Cis-regulatory read out of a Hox/signaling combinatorial code: a mechanism to diversify Hox protein function?

Our study provides a conceptual framework for understanding the molecular basis of regional Hox protein transcriptional activity. We previously reported that Dpp/Tgfβ and Wg/Wnt signaling subdivide the AbdA Hox domain (Bilder et al., 1998), allowing activation of *pointed* (*pnt)* and *opa* target genes in the third and fourth midgut chambers, respectively. Based upon the data presented here, we suspect that the localized activation of *pnt* and *opa* by AbdA also relies on direct enhancer integration of Hox and signaling inputs (Fig. 8). Accordingly, a Hox/signaling combinatorial code functionally

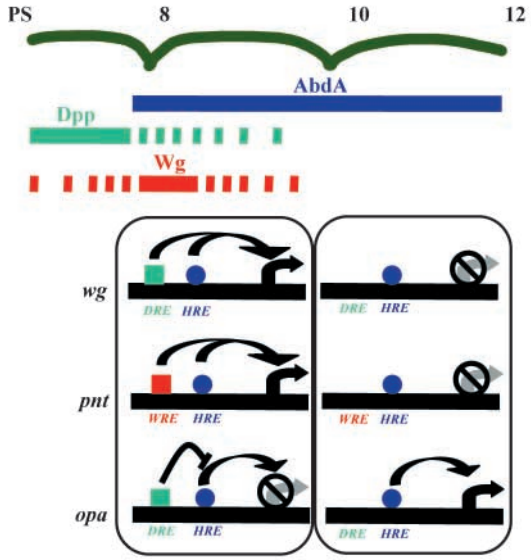

Fig. 8. A model for the regionalization of AbdA activity: cis-regulatory readouts of a Hox/signaling combinatorial code subdivide the domain of AbdA protein function. Simultaneous requirement of Dpp signaling and AbdA results in localized *wg* transcriptional activation in PS8 VM cells. The direct convergence of AbdA and Dpp signaling on a *wg* cis-regulatory region has been established in this study. The model hypothesizes that the regulation of *pnt* and *opa* also results from direct cis-regulatory integration of Hox and signaling inputs. In the regulation of *pnt*, Wg-activated transcription factors synergise with AbdA, ultimately resulting in localized (PS8-10) transcription of *pnt*. In the regulation of *opa* that only occurs in the absence of Dpp signaling, Dpp-activated regulators inhibit *opa* activation through AbdA. According to this model, the cis-regulatory readout of a Hox/signaling combinatorial code is instructive in the regionalization of AbdA transcriptional activity, and thus confers functional diversity to AbdA. WRE, Wg response element; DRE, Dpp response element; HRE, Hox response element.

subdivides the domain where a single Hox protein is made, giving rise to discrete patterns of target gene activation. The structures of relevant cis-regulatory regions of AbdA target genes are instrumental for determining which signal is required to allow activation by AbdA. The *pnt* midgut enhancer would contain AbdA and Wg response elements and would be activated by AbdA specifically in the third midgut chamber through the combinatorial action of AbdA and the *Drosophila* Tcf/Arm transcriptional effector of Wg signaling. Similarly, the *opa* midgut enhancer would contain AbdA and Dpp response elements and would be activated only in the fourth gut chamber by AbdA, in this case because of an inhibitory effect of the Dpp-regulated transcription factor on AbdA activity.

Further studies are required to understand how Hox selector proteins functionally interact with nuclear effectors of signaling pathways to generate specific transcriptional patterns. In the control of *wg* by AbdA, several scenarios can be envisioned. In one, the effect of the Dpp transcriptional effector Mad on AbdA activity would be indirect, by antagonizing the function of a repressor that would otherwise act on the XC enhancer to prevent *wg* expression. The absence of a binding site for this hypothetical repressor in Box2 could explain how Box2 drives AbdA-dependent transcription even without Dpp transcriptional effector binding sites. In a second scenario, Dpp transcriptional effectors would more directly control the activity of AbdA by influencing its DNA binding or transregulatory properties. A direct interaction of HoxC8 and Smad1 has been reported to induce osteoblast differentiation (Shi et al., 1999; Yang et al., 2000), suggesting that the coordinate action of AbdA and Dpp signaling might rely on direct AbdA-Mad interaction. In *wg* regulation, the situation may be different, as additional regulatory inputs are involved. *bin* and *hth* are essential, and Wg signaling is required for accurate levels of *wg* expression. The contribution of Creb might indicate that the Ras/Mapk signaling pathway is involved as well. Ras signaling has been proposed to play a permissive role by acting on CRE sequences of the *Ubx* and *lab* enhancers (Szuts et al., 1998). These observations suggest that AbdA and Hox proteins in general attain specificity and diversity by participating in a variety of protein interactions in enhancer-binding complexes.

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