

***DWnt-4*, a novel *Drosophila Wnt* gene acts downstream of homeotic complex genes in the visceral mesoderm**

Yacine Graba^{*,†}, Kathrin Gieseler[†], Denise Aragnol, Patrick Laurenti, Marie-Christine Mariol, H elene Berenger, Thierry Sagnier and Jacques Pradel[‡]

Laboratoire de G en etique et Physiologie du D veloppement, Institut de Biologie du D veloppement de Marseille, CNRS, Parc Scientifique de Luminy, Case 907, 13288, Marseille Cedex 9, France

^{*}Present address: Department of Developmental Biology, Beckman Centre B 315, Stanford University School of Medicine, Stanford, California 94305-5427, USA

[†]Must be considered as first co-authors

[‡]Author for correspondence

SUMMARY

Wnt genes encode putative cell signalling proteins which play crucial roles during development. From a library of DNA fragments associated, *in vivo*, with Ultrabithorax proteins, we isolated a novel *Drosophila Wnt* gene, *DWnt-4*. Neither a paralog nor an ortholog of the gene exist in the current repertoire of full-length *Wnt* sequences. *DWnt-4* maps close (30 kb) to *wingless*, suggesting that the two *Wnt* genes derive from a duplication that occurred early in evolution, since they are significantly diverged in sequence and structure. Developmental expression of *DWnt-4* partially overlaps that of *wingless*. The gene is transcribed

following a segment polarity-like pattern in the posterior-most cells of each parasegment of the ectoderm, and at two locations that correspond to parasegments 4 and 8 of the visceral mesoderm. The control of *DWnt-4* expression in the visceral mesoderm involves a network of regulatory molecules that includes Ultrabithorax and other proteins from the homeotic complex (HOM-C), as well as the TGF- β *decapentaplegic* gene product.

Key words: cell signalling, *Ultrabithorax* target genes, visceral mesoderm, *Wnt* gene family

INTRODUCTION

In response to the positional information delivered by maternal morphogens and segmentation gene products, the HOM-C selector genes are expressed in defined body parts of the embryo (St Johnston and N usslein-Volhard, 1992; Ingham and Martinez Arias, 1992; McGinnis and Krumlauf, 1992). Their functions are to assign identities and specify the diversified morphogenesis of metameric units (Lewis, 1978). Homeoproteins, acting as sequence-specific transcription factors (Desplan et al., 1988), are thought to achieve their effect by regulating a battery of subordinate target genes (Garcia-Bellido, 1975; Andrew and Scott, 1992). Hence, each metameric unit will enter a specific morphogenetic pathway, depending on the effector target genes regulated by the set of HOM-C genes which are expressed in it. While it is mostly from *Drosophila* that the molecules participating in this genetic cascade have been isolated, growing evidence suggests that the molecular processes involved have their counterparts in vertebrates (Duboule, 1992; McGinnis and Krumlauf, 1992; Bachiller et al., 1994).

Most of the few putative targets of HOM-C genes identified so far encode regulatory molecules, thus demonstrating further complexity in the genetic cascade upstream of the 'realizator' genes whose products act directly in morphogenesis and differ-

entiation processes (Morata, 1993). Besides genes that themselves encode transcriptional regulators, several are involved in cell-cell communication, and presumably therefore in signal transduction pathways downstream of which transcription factors function (Botas, 1993). The products of two candidate targets identified using immunopurification procedures, *connectin* (Gould et al., 1992) and *scabrous* (Graba et al., 1992), function as cell signalling molecules. *connectin* encodes a cell surface protein which acts as a homophilic cell-cell adhesion protein and probably has a role in the formation of neuromuscular connections (Nose et al., 1992). *scabrous* protein is thought to be involved in lateral inhibition during the development of the nervous system (Baker et al., 1990). Two more genes, *decapentaplegic* (*dpp*) and *wingless* (*wg*), encode secreted glycoproteins whose expression is regulated in the visceral mesoderm (VM) by the action of homeotic proteins (Immergl uck et al., 1990; Reuter et al., 1990; Capovilla et al., 1994). *dpp* is a member of the TGF- β family (Padgett et al., 1987) and *wg* is a segment polarity gene, which belongs to the *Wnt* family (Rijsewijk et al., 1987).

A variety of *Wnt* genes has now been discovered in various species (Nusse and Varmus, 1992; McMahon, 1992; Sidow, 1992). Every *Wnt* deficiency or ectopic expression provokes dramatic developmental defects, in the morphogenesis of the central nervous system (McMahon and Bradley, 1990; Thomas

and Capecchi, 1990), cell proliferation (Roelink et al., 1990; Skaer and Martinez Arias, 1992), embryonic axis specification and mesodermal patterning (McMahon and Moon, 1989; Takada et al., 1994), embryonic segmentation (Nüsslein-Volhard and Wieschaus, 1980; Noordermeer et al., 1992) or limb patterning (Couso et al., 1993; Struhl and Basler, 1993). Wnt proteins are thus recognised as cell signalling mediators that play important regulatory functions in a number of basic developmental processes.

Orthologs of the murine *Wnt-5* and *Wnt-7*, have been recently identified in *Drosophila* (Eisenberg et al., 1992; Russel et al., 1992). In this paper, we report the characterisation of a novel *Drosophila* *Wnt* gene, cloned from a library of genomic DNA fragments associated in vivo to Ubx proteins (Graba et al., 1992). This gene locates cytogenetically to the same chromosomal position as *wg*, the 5' ends of their translated regions being separated by 30 kb of DNA. It shows a segment polarity-like pattern of expression in the ectoderm and is controlled by HOM-C genes in the VM.

MATERIALS AND METHODS

Flies and egg collection

Oregon R was used as the wild-type standard. The mutant alleles used were *Scr^{W17}* (Wakimoto and Kaufman, 1981), *Antp^{W10}* (Wakimoto and Kaufman, 1981), *Ubx⁹²²* (Kerridge and Morata, 1982), *abd-A^{MX1}* (Sanchez-Herrero et al., 1985), *Df(3R)109* (Lewis, 1978), *dpp^{shv4}* (Segal and Gelbart, 1985) and *dpp^{shv6}* (Segal and Gelbart, 1985). The *Antp-Ubx* chromosome was generated by recombination using the *Antp^{W10}* and *Ubx⁹²²* alleles.

Flies were grown in standard culture conditions at 25°C. Egg collections were made from transheterozygotes over a wild-type chromosome, dechorionated in 50% bleach for 2 minutes, prefixed in a mix (v/v) of n-heptane and PBS buffer containing 50 mM EGTA, 10% formaldehyde, devitelinised in a -70°C prechilled mix of n-heptane/methanol (v/v) containing 50 mM EGTA, and stored in ethanol at -20°C.

In situ hybridisation to whole embryos and polytene chromosomes

In situ hybridisation to whole embryos using digoxigenin-labelled probes (Boehringer) was performed according to the method of Tautz and Pfeifle (1989). To detect *DWnt-4* RNA, we used either a 4.2 kb *EcoRI* genomic fragment, which overlaps 1.4 kb of the *DWnt-4* transcribed region, or the 3.2 kb *DWnt-4* cDNA. To detect *wg* RNA, we used a 1 kb fragment lying in the 5' coding sequence of the gene. After phosphatase alkaline detection, embryos were mounted in 90% glycerol, 100 mM Tris, pH 7.5 and observed under an AxioPhot Zeiss microscope using Nomarski optics. In situ double labelling experiments were performed according to the method of Röder et al. (1992), using the monoclonal antibody 4D9 (Patel et al., 1989) to visualise the En stripes. In situ hybridisation to a polytene chromosome was performed using biotinylated probes and the Vectastain peroxidase detection kit (Vector Laboratories).

Isolation of genomic DNA and cDNA clones

Phage (Maniatis et al., 1978) and cosmid (Hoheisel et al., 1991) *Drosophila* genomic libraries were screened with clone CU83 and DNA fragments 5' to *wg*. Overlapping clones (4 phages and one cosmid) spanning 64 kb were recovered. *DWnt-4* RNA was identified on northern blots probed with the 4.2 kb *EcoRI* fragment located at the left end of the λ 83.1 phage (Fig. 1A). We failed to detect any other transcript within the intercalating DNA between *wg* and *DWnt-4*.

Eight overlapping cDNA clones were recovered by probing a 4 to 8 hour embryonic cDNA library (Brown and Kafatos, 1988) with the 4.2 kb *EcoRI* fragment.

Sequence determination and analysis

The longest cDNA clone (3.2 kb) was subcloned in both orientations into the Bluescript vector. Nested deletions of the insert fragments were generated by digestion with *ExoIII* and the nucleotide sequence (on both strands) was determined using Sequenase 2.0 kit (US Biochemicals). A fragment of 277 bp, which corresponds to the 3' end of *DWnt-4* coding sequence, was amplified from genomic DNA using the primers: 5'-CGCAATACACCACGCTGTAC and 5'-AAGTCT-GCGAGAGATGGCAC. The amplified fragment was subcloned in the PCRII plasmid (Invitrogen) and sequenced.

The alignment of amino acid sequences of *Drosophila* Wnt proteins was constructed with the Geneworks Alignment Program (Intelligenetics Inc.) and refined by hand in order to minimise the 'cost' given by an algorithm similar to FASTA.

The phylogenetic tree was determined without taking into account the sequences upstream of the first conserved cysteine and obvious inserted sequences. This corresponds in Wg to the deletion of amino acids 1-57, 167-178, 205-208, 293-373. Homothetic deletions were made in the other Wnt sequences. These truncated sequences were then aligned as described above, and a phylogenetic tree constructed using the unweighted pair group method with an arithmetic mean (UPGMA-Tree Program, Geneworks, Intelligenetics Inc.).

Gel mobility shift assay

The Ubx fusion protein construct in the T7 polymerase expression vector pET3a was kindly provided by R. A. H. White (University of Cambridge). Heparine-agarose purified Ubx protein extracts were separated by 12.5% SDS-PAGE, the fusion protein cut out from the gel and electroeluted. The purified Ubx protein was dialysed for 4 hours against a denaturing buffer (25 mM Hepes, 100 mM KCl, 0.1% NP40, 6 M Urea) and overnight in a renaturing buffer (25 mM Hepes, 100 mM KCl, 1 mM PMSF, 1 mM EDTA, 1 mM DTT and 20% glycerol, pH 7.9), and stored in small aliquots at -80°C.

DNA-protein interactions were performed in 10 mM Hepes, 0.1 mM EDTA, 30 mM KCl, 1 mM MgCl₂, 0.1 mg/ml BSA, pH 8.0. Ubx protein was preincubated in a 20 μ l reaction volume for 30 minutes on ice. 10⁴ cpm of ³²P-labelled CU83 fragment was added and the incubation continued for 30 minutes on ice. The formation of DNA protein complexes was detected by autoradiography after 5% PAGE (20 mA) performed under non-denaturing conditions (TBE). In super shift assays, FP3.38 monoclonal antibody (50 ng) was added to Ubx fusion protein during the preincubation step of 30 minutes, before the addition of CU83-labelled fragment.

RESULTS

Cloning the *CU83* locus

We previously reported a library of genomic DNA fragments associated in vivo with Ubx homeoproteins (Graba et al., 1992). In a further screen of this library, we selected a clone, CU83, which is particularly abundant, representing about 0.8% of the total clones. The nucleotide sequence of CU83 (127 bp) revealed the presence of a motif (underlined in Fig. 1A) containing the ATTA core motif found in most homeodomain binding sites (Desplan et al., 1988). This motif, TTAATTAGC, resembles the consensus TTAAT(G/T)(G/A)CC defined by Ekker et al. (1992) as the optimal DNA sequence recognised by the Ubx homeodomain in vitro and hence suggests that the gene associated with the fragment represents a bona fide target of homeoproteins. Gel shift assays confirm this (Fig. 2) and

demonstrate that Ubx protein efficiently binds clone CU83, *in vitro*.

Genomic DNA from the locus was recovered using CU83 to screen genomic libraries (Fig. 1). Probing northern blots of 5 to 15 hour embryonic mRNA with all the *EcoRI* fragments of phage λ 83.1, identified a single 3.2 kb transcript, 7 kb away from clone CU83 (not shown). Furthermore, by probing an embryonic cDNA library constructed by Brown and Kafatos (1988), we isolated 8 overlapping cDNA clones of the corresponding gene, which we also named *CU83*.

CU83 was localised, by *in situ* hybridisation, at 28A on the left arm of chromosome 2 (not shown), i.e. at the same chromosomal position as *wg*. We mapped it more precisely within a chromosomal walk initiated from probes 5' to *wg* and *CU83*. The translation starts in *wg* and *CU83* are separated by 30 kb of DNA. On the basis of restriction analysis and polymerase chain reaction (PCR) amplification data, two introns of 18 kb and 300 bp were identified and localised in the coding of the gene (Fig. 1B).

Sequence determination and predicted protein

The complete nucleotide sequence of the longest cDNA is available from data banks. Comparison of its length (3201 bp) with the size of the mRNA detected by northern blot (3.2 kb) suggests that all of the sequence of the *CU83* transcript has been recovered. The longest open reading frame (ORF) extends 1167 bp. The sequence surrounding the first methionine codon of the ORF at nucleotide 1492, 5'-CAACATGG-3', matches the consensus, 5'-(A/C)AA(A/C)-ATGG-3', for translation start in *Drosophila* (Cavener, 1987). This ATG is the first methionine codon preceded in frame by several stop codons in the cDNA sequence (at position 946, 1018 and 1039). An AATAAA poly(A) addition signal is present at position 3147, near the 3' end of the untranslated region.

The deduced protein sequence, shown together with those of Wg, DWnt-2 and DWnt-3 in Fig. 3A, is highly homologous to the members of the *Wnt* gene family. Accordingly, the *CU83* gene will be now referred to as *DWnt-4*. A signal sequence (Von Heijne, 1985), from residue 1 to 27, as well as putative sites for N-linked glycosylation predict a secreted glycoprotein. The

most significant feature of Wnt proteins is an abundance of strongly conserved cysteine residues (Nusse and Varmus, 1992; Sidow, 1992). Besides the 22 cysteines found in all Wnt sequences, DWnt-4 contains two residues, at position 90 and 103, which exist in all proteins other than Wnt-1 orthologs. It lacks the cysteine unique to Wnt-1 (position 124 in Wg). Another rule among Wnt proteins is that the spacing between the conserved cysteines is largely invariant. In three places in the carboxy-terminal part of DWnt-4 this rule is not satisfied. Relative to the other family members, DWnt-4 has 2 extra amino acids between cysteines 19 and 20 and two gaps of 8

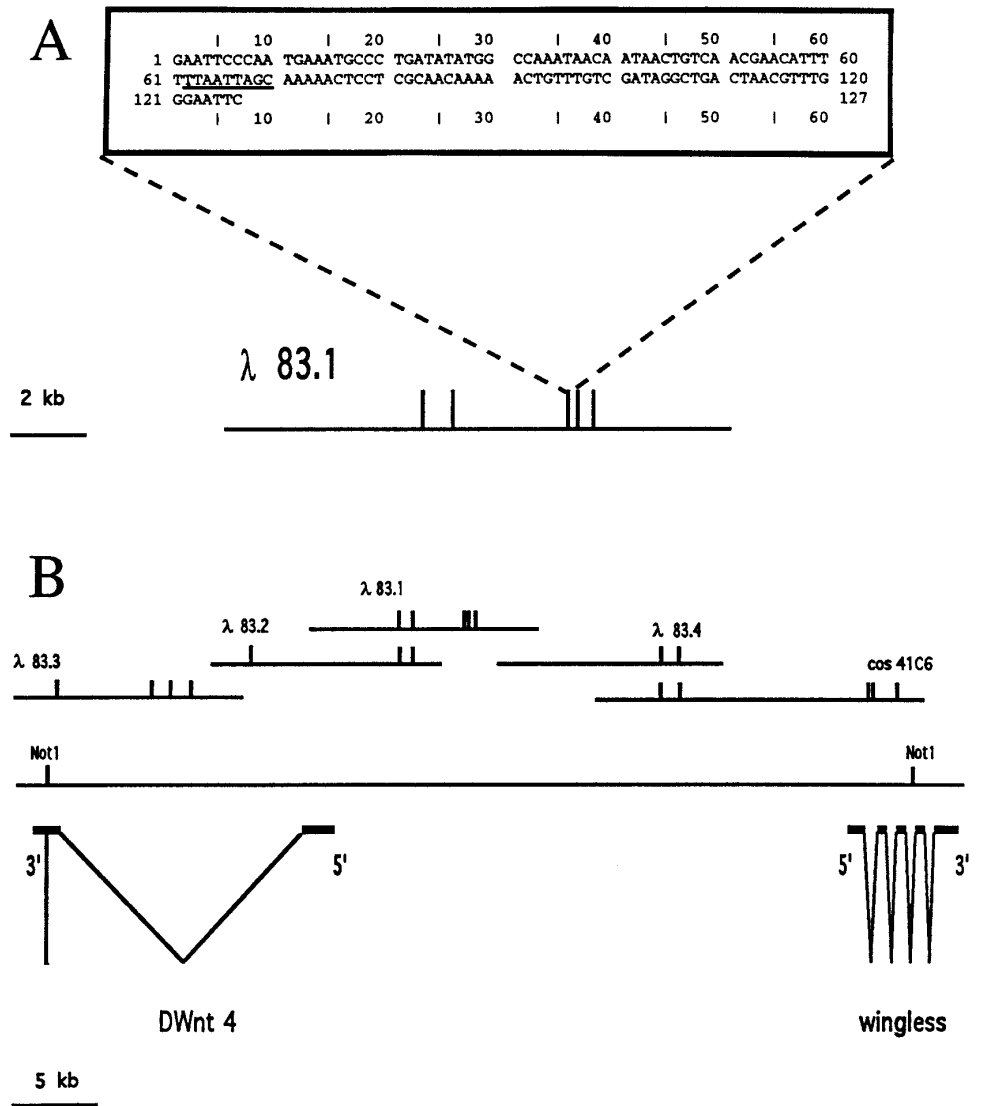


Fig. 1. Cloning the *CU83* locus. (A) Clone *CU83*. The motif *TTAATTAGC*, underlined in the sequence of *CU83* (127 bp), closely resembles the consensus *TTAAT(G/T)(G/A)CC* given by Ekker et al. (1992) as the optimal recognition site of Ubx proteins. DNA from phage λ 83.1, isolated by probing a genomic library with the clone *CU83*, is shown with *EcoRI* sites depicted as small vertical bars. Only the 4.2 kb *EcoRI* fragment (at the left end of λ 83.1) reacted in northern blot experiments. (B) Molecular map of the *wg/DWnt-4* locus. *EcoRI* sites are indicated as vertical bars on phage and cosmid DNA. The contig obtained from overlapping phages and cosmid places the *NotI* sites present in *wg* and *DWnt-4* 60 kb one from another. Consistently, a *NotI* fragment of the same size is recognised on a Southern blot of genomic DNA separated by pulsed field gel electrophoresis (not shown). *wg* and *DWnt-4* transcription units are displayed below.

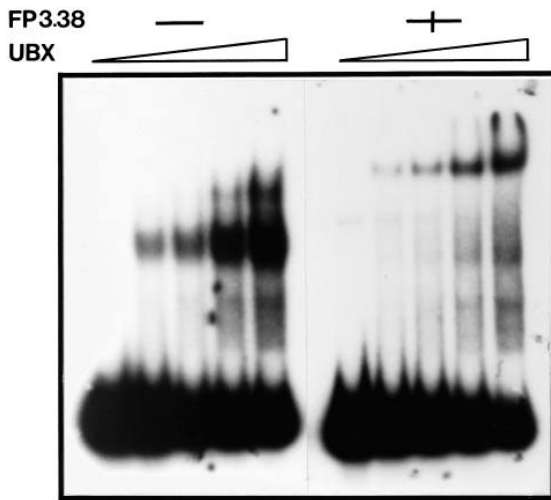


Fig. 2. Clone CU83 binds Ubx in vitro. Increasing amounts of Ubx (0, 0.5, 1, 2 and 4 ng) in the absence (left side) and in the presence (right side) of FP3.38 anti-Ubx antibody were incubated with ^{32}P -labelled CU83 and a gel shift mobility assay was used to display the products. Note the formation of DNA-Ubx complexes on the left side and the supershifted bands on the right.

and 4 amino acids between cysteines 13/14 and cysteines 14/15, respectively. We confirmed the primary structure of the C terminus of DWnt-4 by sequencing a genomic DNA fragment spanning the 3' coding region (from nucleotides 2403 to 2680).

On the whole, the DWnt-4 sequence is 35% identical to the Wg sequence, 32% to DWnt-2, and 33% to DWnt-3, not taking into account the non-conserved amino termini and the inserts found in each protein. Comparison with Wnt proteins from other species gave identity scores also ranging from 30% to 35%. The dendrogram of Fig. 3B, which provides a phylogenetic relationship between mouse and *Drosophila* Wnt proteins, puts DWnt-4 in a branch distinct from the others. Thus, DWnt-4 is not orthologous to a known vertebrate Wnt gene nor paralogous to a *Drosophila* Wnt gene. Interestingly, out of the 59 predicted C-terminal regions of Wnt proteins (from cysteines 11 to 19) identified by Sidow (1992), Wnt-9 sequences from hagfish (*Eptatretus stouti*) and shark (*Alopius vulpinus*) are the only ones that show an unusual structure with gaps at similar positions to those in DWnt-4, between cysteines 13/14 and 14/15 (Fig. 3C). It must be noted that Wnt-9 conservation between species separated by more than 500 millions years (hagfish and shark), means that the genes are functional. While the comparison has so far only been made on partial sequences, DWnt-4 could yet constitute the *Drosophila* ortholog of vertebrate Wnt-9.

DWnt-4 transcription during embryogenesis

Transcripts are first detected in the ectoderm of stage 8 embryos at the anterior tip and in the trunk, where a segmentally repeated striped pattern becomes visible ventrally (Fig. 4B). Shortly afterwards, expression in the head region is seen in the foregut primordium and dorsal patches that presumably correspond to cells of labral and ocular segment primordia (Fig. 4C). By completion of germ band extension, the segment

polarity-like staining in the trunk intensifies and 15 stripes are clearly seen in parasegments (PS) 0 to 14 (Fig. 4C). Double staining for Engrailed (En)/Invected (Inv) proteins as well as *wg* or DWnt-4 transcripts indicate that stripes are one cell wide and overlap the ventralmost *wg* cells (Fig. 5).

Before germ band retraction, the DWnt-4 pattern in the trunk ectoderm evolves. Transcripts become visible in the dorsolateral ectoderm of each PS, in a short row of 3-4 cells which parallels the En stripe but which is located at the middle of the PS (arrow in Fig. 5A). At the posterior tip of the germ band, a complex expression is initiated with one additional stripe posterior to the En stripe, which marks the anterior border of PS14, and a strong staining of the telson (Figs. 4F, 5C).

During germ band shortening, the striped staining in the ectoderm slowly diminishes in intensity and fades at the end of the process. By this time, transcripts become detectable in the central nervous system and developing gut. Expression in the nerve cord is not uniform, but instead increases progressively from the anterior to posterior regions of the embryo (Fig. 4G, I). In the gut, transcripts are seen in foregut, hindgut and at two locations in the VM of the midgut (Fig. 4H). The anteriormost domain, which is separated from the foregut by 3-4 unlabelled cells, presumably corresponds to PS4. The second domain was localised through double-label experiments for DWnt-4 transcripts and Ubx or En proteins (not shown). The landmarks provided by Ubx in PS7 of the VM (Bienz and Tremml, 1988) and the En stripes indicated that the posteriormost domain corresponds to PS8. The two domains of DWnt-4 transcription in the VM coincide with primordia of the gastric caeca and the second midgut constriction. Later, the gene is clearly expressed at, and adjacent to, the sites of these differentiative events (Fig. 4J).

Three HOM-C genes and *dpp* control DWnt-4 in the VM

Four HOM-C genes, expressed in exclusive parasegmental domains of the VM, control gut morphogenesis: *Sex combs reduced* (*Scr*), *Antennapedia* (*Antp*), *Ubx* and *abdominal-A* (*abd-A*) in posterior PS3 to PS4, PS5 to PS6, PS7, and from PS8 to PS12, respectively (Tremml and Bienz, 1990; Reuter and Scott, 1990). Since DWnt-4 was originally identified as a putative target of Ubx, we investigated whether its transcription in the VM might depend on Ubx and other HOM-C gene activities.

In embryos deficient for *Ubx* or *abd-A*, expression in PS8 is absent (Fig. 6B,C). Thus, *Ubx* in PS7 and *abd-A* in PS8 are necessary for DWnt-4 activation in PS8. This is reminiscent of the regulation of *wg* by HOM-C genes in the VM, and suggests a positive and direct control by *abd-A* and a positive but indirect control by *Ubx*. We examined whether the secreted Dpp protein, which mediates the action of Ubx on *wg* in PS8 of the VM (Immerglück et al., 1990), might also control DWnt-4 expression. In the *shortvein* class of mutations (*dpp^{shv}*), *dpp* expression, which normally occurs in PS4 and PS7 of the VM, is disrupted either in one or both domains (Hursh et al., 1993). In *dpp^{shv6}* embryos, which lack *dpp* activity in PS7, we fail to detect DWnt-4 transcripts in PS8 (Fig. 6D). In the *dpp^{shv4}* embryos, which lacks *dpp* expression in PS4 and in PS7, transcripts are no longer detected in the VM, either in PS4 or in PS8 (Fig. 6E). *dpp* is therefore required for DWnt-4 activation not only in PS8, as it is for that of *wg*, but also in the anterior

part of the midgut (PS4). *Scr* is dispensable for *DWnt-4* regulation since in the null mutant *Scr^{w17}*, the gene is still expressed in PS4 (not shown).

In *Antp* deficient animals, *DWnt-4* becomes ectopically expressed in PS5 and PS6 (Fig. 6F), indicating a negative regulation by *Antp* protein in this portion of the VM. Regulatory interactions between HOM-C genes occur in the VM as well as in the epidermis (Bienz and Tremml, 1988; Reuter and Scott, 1990); for instance, *Ubx* sets the posterior border of *Antp* expression such that, in *Ubx* mutants, *Antp* is derepressed posteriorly in PS7. We asked whether the ectopic expression of *Antp* could silence *DWnt-4* in PS7 of *Ubx* homozygotes. If so, *Antp* would replace *Ubx* as a repressor of *DWnt-4* in this PS; in other words, the derepression of *DWnt-4* in PS7 resulting from the absence of *Ubx* activity would be masked by the ectopic accumulation of *Antp*. One would therefore expect *DWnt-4* to be expressed not only in PS5-6 but also in PS7 of embryos deficient for both *Antp* and *Ubx* functions. Indeed, transcription in these embryos extends posteriorly to include PS7 (Fig. 6G). Note that expression is clearly absent in PS8, which is likely to be due to the lack of *dpp* activation by *Ubx*. Thus, ectopic *Antp* is able to repress *DWnt-4* in PS7 of embryos lacking *Ubx* function, a strong indication that the down-regulation, in PS7 of wild-type embryos, is achieved by *Ubx*. Comparison of *DWnt-4* patterns in PS7 and PS8 of *Antp*, *Ubx* and *Antp-Ubx* mutants, illustrates the dual regulatory function of *Ubx* which in the wild type turns *DWnt-4* off in PS7 and on in PS8.

In *abd-A* mutants, *Ubx* expression extends to the posterior end of the midgut (Tremml and Bienz, 1989). To test whether this ectopic expression could have an effect, we analysed the *DWnt-4* pattern in embryos deficient for both *Ubx* and *abd-A*. In such embryos, as the ectopic expression of *Antp* does not extend back to PS7 (Bienz and Tremml, 1988), none of the HOM-C genes are expressed from PS9 to PS12 of the VM. We found that *DWnt-4* became ectopically expressed from PS9 to PS12 (Fig. 6H). The simplest explanation is that *abd-A* silences *DWnt-4* in this portion of the VM of wild-type embryos and that, in the absence of *abd-A* function, repression is achieved by *Ubx*. These data clearly illustrate that *abd-A* also achieves

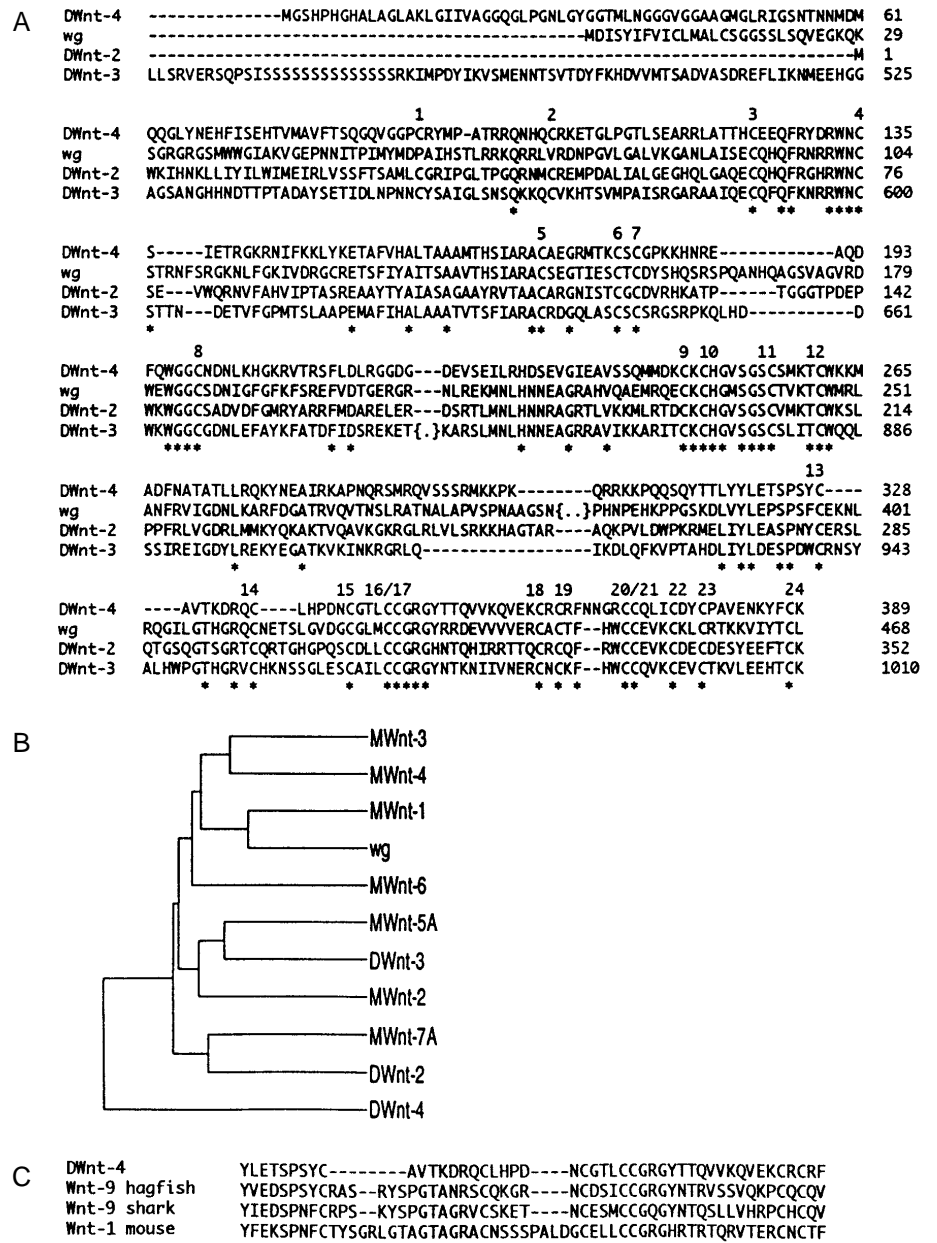


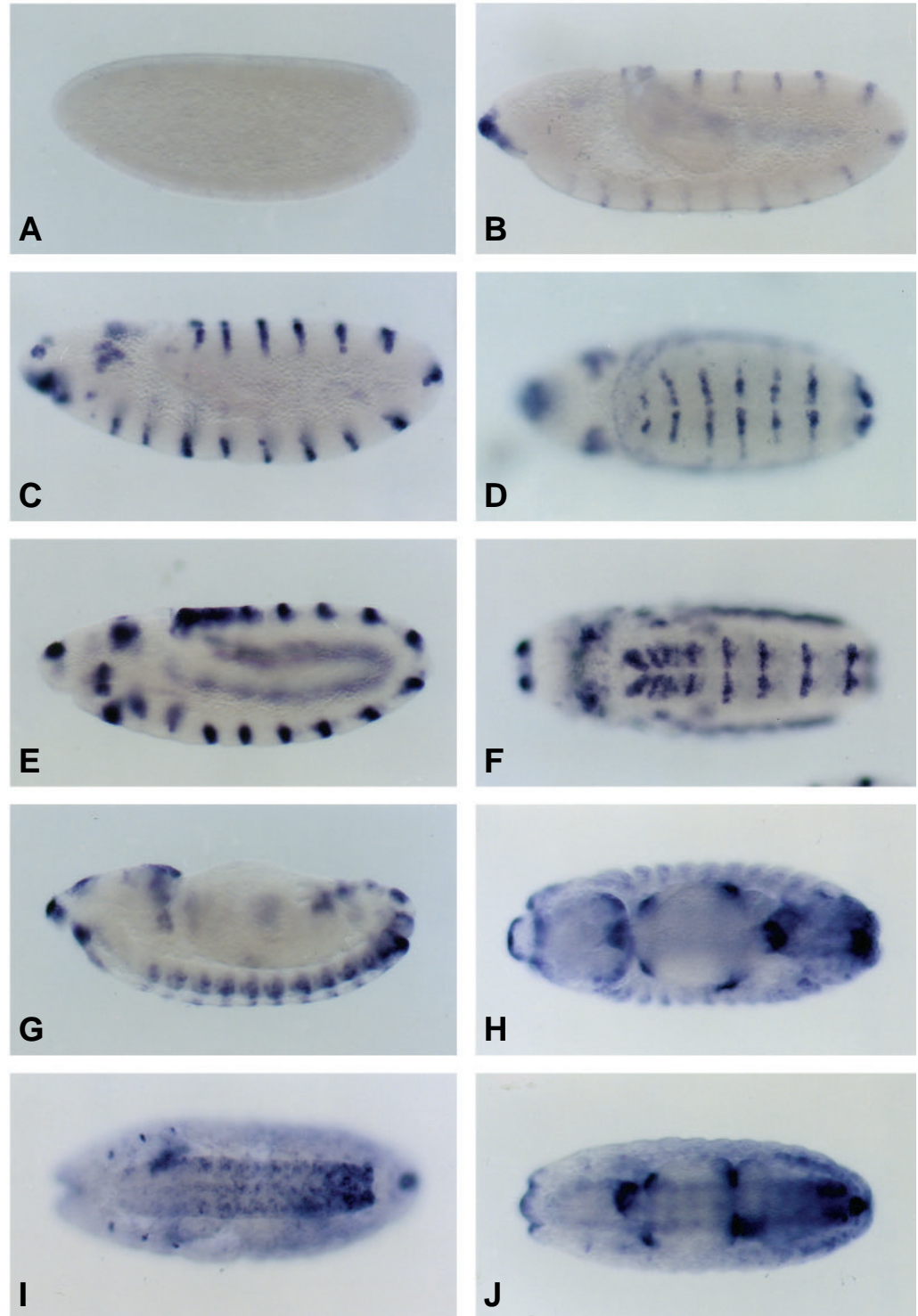
Fig. 3. *CU83* is a member of the *Wnt* family. (A) Alignment of deduced amino acid sequences of the *DWnt-4* gene product and the *Drosophila* Wg, DWnt-2 and DWnt-3 proteins. Regions with no significant Wnt homology have been deleted: in DWnt-3, the first 450 amino acids and a 153 amino acid insert, indicated by {..}, between positions 689 and 842; in Wg, a 79 amino acid insert, indicated by {..}, between positions 294 and 373. Asterisks indicate the amino acids that are conserved in the four *Drosophila* Wnt sequences, with the cysteine residues numbered from 1 to 24. Note the unusual feature of the C terminus of DWnt-4 with two deletions of 8 and 4 amino acids between cysteines 13/14 and cysteines 14/15, and the insertion of 2 extra amino acids between cysteines 19/20. (B) Graphical representation of the sequence relationship of mouse and *Drosophila* Wnt proteins. The tree, constructed using the UPGMA method (see Materials and Methods section), puts the *Drosophila* sequences together with their respective orthologs (Wg/Wnt-1; DWnt-2/Wnt-7; DWnt-3/Wnt-5) and indicates that DWnt-4 has neither an ortholog nor a paralog in the current Wnt repertoire. (C) Alignment of the C-terminal parts of Wnt-9 fragments from hagfish (*Epiplatys stouti*) and shark (*Alopius vulpinus*) with corresponding regions of mouse Wnt-1 and DWnt-4. Notice the gaps at similar positions between cysteines 13/14 and 14/15 in the Wnt-9 and DWnt-4 sequences.

a dual regulation of *DWnt-4* in the VM, positive in PS8 and negative from PS9 to PS12.

The strong and complex expression of *DWnt-4* seen in the ectoderm at the posterior tip of the germ band (Fig. 4F) as well as in the posterior region of the nerve cord during germ band

retraction (Fig. 4I), seems to be related to that of *Abdominal-B* (*Abd-B*). Analysing these patterns in several mutant backgrounds lacking the *Abd-B* function (*Abd-B*⁻, *abd-A*⁻*Abd-B*⁻, *Antp*⁻*Ubx*⁻*abd-A*⁻*Abd-B*⁻), we do not observed any change relative to the wild type. It appears therefore that firstly, *Abd-*

Fig. 4. Spatial distribution of *DWnt-4* transcript during embryogenesis. Embryos (anterior to the left, ventral side down in lateral views) were staged according to Campos-Ortega and Hartenstein (1985). (A) Lateral view of a cellular blastoderm embryo (stage 5). *DWnt-4* transcripts are not detected at this stage. (B) Same orientation of an embryo in rapid phase of germ band elongation (stage 8). *DWnt-4* transcripts appear at the anterior tip of the embryo and follow a segment polarity-like pattern in the trunk epidermis. (C,D) Embryos at fully extended germ band stage (stage 10). The segment polarity-like pattern is now well established with 15 stripes corresponding to PS0-PS14. In the head region, transcripts are seen in the foregut primordium and dorsal patches, that presumably correspond to the labral and ocular segment primordia (C). The dorsal view (D) indicates that transcripts are detected in the ventralmost ectodermal cells in each PS. Stripes are one cell wide. (E,F) Embryos at the onset of germ band retraction (stage 11). A complex expression appears at the tip of the germ band, with an additional stripe posterior to PS14 (see also Fig. 5C). Note also that expression is initiated in the dorsolateral ectoderm (out of focus here, see Fig. 5A). (G,H,I) Embryos after completion of germ band shortening (stage 14-15). While expression in the ectoderm fades, transcripts become visible in the developing gut and the nerve cord. In the gut, transcripts are detected in the foregut, hindgut and at two locations, approximately one segment wide each, in the VM (lateral and ventral views in G and H, respectively). The anterior domain in the VM is separated



by 3-4 unlabelled cells from the foregut and presumably corresponds to PS4. The posterior domain corresponds to PS8. In a slightly older embryo, staining in the nerve cord intensifies, revealing an increasing transcript distribution from anterior to posterior (I). (J) Stage 16 embryo showing the *DWnt-4* RNA localised in VM cells within the anlage of the gastric caeca and the second midgut constriction.

B does not play on *DWnt-4* in the caudal region of the embryo, and secondly, regulation by homeotics takes place in one germ layer only, the VM.

The regulatory network composed of *dpp* and HOM-C genes that controls *DWnt-4* expression in the VM is schematised in Fig. 7.

DISCUSSION

The identification of genetic functions acting downstream of HOM-C genes is of central importance in the understanding of the homeotic control of regional identity and morphogenesis. The immunoselection of DNA fragments bound to homeoproteins *in vivo* constitutes an approach specially designed to clone direct effectors of homeotic genes. *DWnt-4*, isolated from a further screen of a library of Ubx-associated DNA fragments (Graba et al., 1992), presents several features shared by most of the putative targets identified so far (Botas, 1993). First, the gene is regulated by several HOM-C genes; its pattern of expression in the VM results from regulatory interactions between the homeotic genes active in this germ layer. Second, in keeping with the expression pattern, *DWnt-4* presumably is involved in developmental processes other than downstream of HOM-C genes. Third, the identification of *DWnt-4* as a putative target of homeotic proteins is a further indication that effector genes encode molecules involved in growth and cell fate decisions rather than, so far, housekeeping functions.

DWnt-4, a new Wnt gene in the *wg* locus

DWnt-4 encodes a protein with features characteristic of Wnt gene products. First, the amino-terminal signal sequence and prospective sites for N-linked glycosylation predict a glycoprotein destined for secretion. Second, the 24 cysteines in the *DWnt-4* sequence correspond to conserved residues found in all Wnt proteins other than Wnt-1. Based on the conservation of cysteines as a criterion for the relatedness of the Wnt genes, *DWnt-4* is less related to Wnt-1, Wg in *Drosophila*, than to the other Wnt proteins. In terms of overall conservation of amino acid residues, homology scores with the other family members, from *Drosophila* as well as from widely divergent species, range around 30-35%. It appears therefore that neither a paralog nor an ortholog of this gene exists within the current repertoire of full-length Wnt sequences. Comparison with 59 Wnt partial sequences revealed that the C terminus of vertebrate Wnt-9 and *DWnt-4* show the same unusual structure, with gaps at similar positions between conserved cysteines. This suggests that the corresponding genes could be orthologous. The connection with vertebrate Wnt-9 indicates that *DWnt-4* presumably does not arise from a gene duplication in some ancestral arthropod, but much

earlier, before the divergence of deuterostome and arthropod lineages.

Several loci consisting of related genes have been described. Generally, expression patterns are virtually identical or largely overlapping and the predicted proteins show considerable homology, at least in the functional domains; in some cases, it has been demonstrated that the genes are functionally related (e.g. Rothe et al., 1992; Grossniklaus et al., 1992; Zhang et al., 1994). *wg* and *DWnt-4* are not paralogous Wnt genes and have distinct intron-exon structures, consistent with the previous proposal that the genes diverged early in evolution. Their expression profiles, while related, differ in several respects. Both genes are transcribed in the same ventral cells of the posteriormost ectoderm of each PS, in PS8 of the VM, in the head region and in foregut and hindgut primordia; but they are also found in specific domains: *wg* and *DWnt-4* in distinct cell rows of the dorsolateral ectoderm, *wg* in Malpighian tubules, and *DWnt-4* in the nerve cord and PS4 of the VM. The close physical linkage, coexpression and coregulation suggest that the two Wnt genes share cis-control elements and belong to the same gene networks in developmental processes. Supporting this view, we isolated several EMS induced regulatory mutations that affect expression of the two genes; moreover, while the absence of an amorphous *DWnt-4* mutation prohibits us from conclusively demonstrating a function in segmental patterning and gut morphogenesis, we identified a hypomorphic *DWnt-4* allele which has a segment polarity phenotype, indicating a function in embryonic epidermis (not shown).

DWnt-4, a new signalling molecule downstream of HOM-C genes and *dpp* in the VM

The homeotic proteins Antp, Ubx and Abd-A control, directly or indirectly, *DWnt-4* expression in several portions of the VM. This regulatory network, which also includes Dpp, leads to

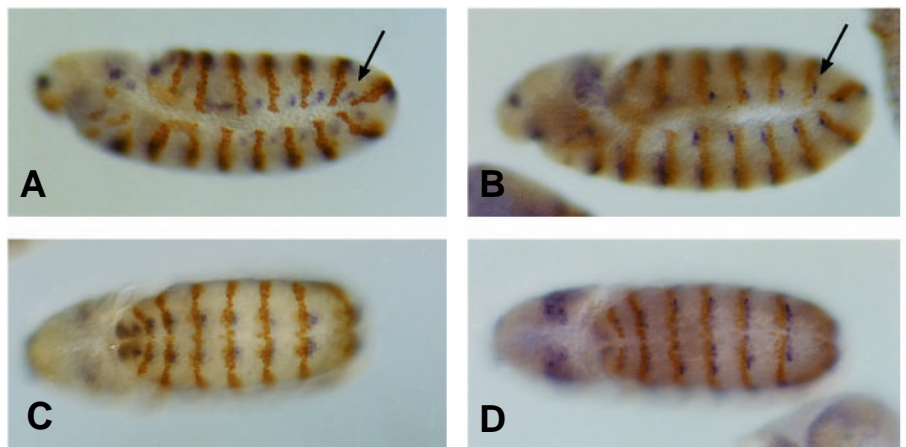


Fig. 5. Intrasegmental localisation of *DWnt-4* transcript. Embryos (anterior to the left) were doubly labelled for En/Inv protein and for *DWnt-4* (A,C) or *wg* (B,D) RNA. The En/Inv proteins (in brown) mark the two anteriormost cell rows of each PS and serve as a reference for transcript localisation (in blue). A and B are lateral and D and E are ventral views of embryos at the onset of germ band retraction (late stage 11). In dorsolateral ectoderm, while *wg* is expressed in cells along the PS border (arrow in B), *DWnt-4* RNA is detected in a cell row located near the middle of the PS (arrow in A). In the ventral ectoderm, *DWnt-4* (C) and *wg* (D) are expressed in posteriormost cells of each PS, *DWnt-4* transcripts being detected in the ventralmost *wg* cells only. Note at the tip of the germ band in C an additional *DWnt-4* stripe, posterior to the 14th En stripe.

DWnt-4 expression at two locations in the VM where major morphogenetic events occur: the formation of the gastric caeca and the second midgut constriction.

Evidence for a direct relationship exists only for *Ubx* and clone CU83: the clone has been selected from the library of *Ubx* targets, it presents an ATTA nucleotide motif required for homeodomain association and actually binds *Ubx* in vitro.

Accordingly, the interaction between *Ubx* and the 127 bp element could be responsible for the *DWnt-4* silencing observed in vivo in PS7 of the VM. The gene is also down-regulated by *Antp* and *abd-A* in PS5-6 and PS9-12, respectively. Moreover, ectopic *Antp* in PS7 and *Ubx* in PS9-12 repress *DWnt-4*. Comparable situations, where HOM-C proteins can be replaced by one another to achieve repression,

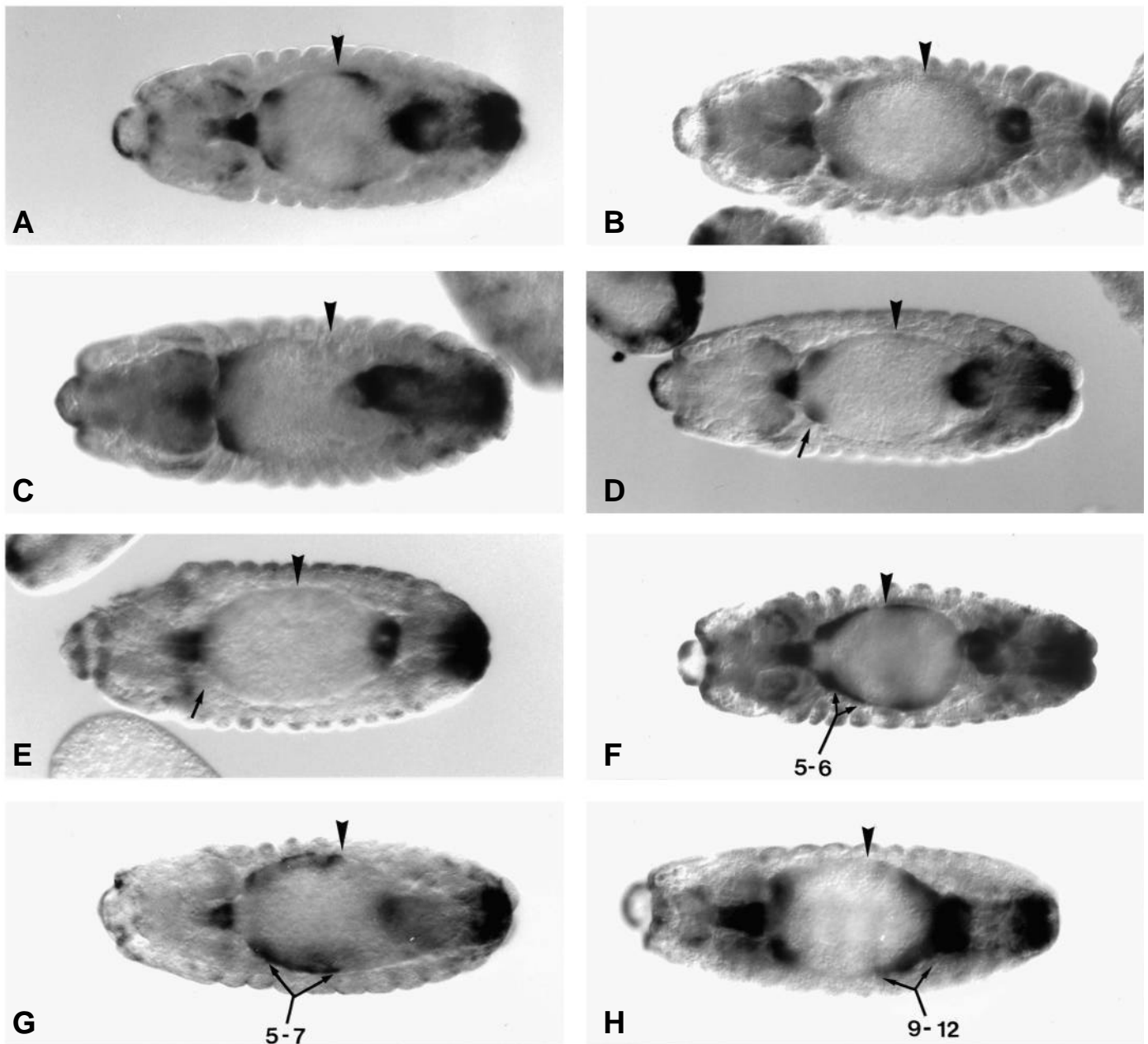


Fig. 6. *DWnt-4* regulation in the midgut. All photos are ventral views of stage 15 embryos (anterior to the left) hybridised with a *DWnt-4* probe. Arrowheads point towards the posterior boundary of PS7 in the VM. (A) Wild-type embryo. *DWnt-4* transcripts are detected in PS4 and PS8 of the VM. (B,C) Embryos deficient for *Ubx* and *abd-A*, respectively. Note the absence of *DWnt-4* transcript in PS8 in both mutants. (D,E) Embryos homozygous for *dpp shortvein* mutations. In *dpp^{shv6}*, *DWnt-4* RNA is seen in PS4 (arrow in D) but not in PS8. In *dpp^{shv4}*, *DWnt-4* transcription is no longer detected in the VM, either in PS4 (arrow in E) or in PS8. (F) In a mutant deficient for *Antp*, *DWnt-4* becomes ectopically transcribed in PS5 and PS6 (arrows). (G) In a double mutant for *Antp* and *Ubx*, the ectopic expression of *DWnt-4* invades PS7 (arrow). Note that, as in a *Ubx* single mutant, transcripts are not detectable in PS8. (H) In embryos deficient for *Ubx* and *abd-A*, ectopic *DWnt-4* RNA is seen in PS9-12, which corresponds to the posterior part of the *abd-A* expression domain. As in an *abd-A* single mutant, expression in PS8 is missing.

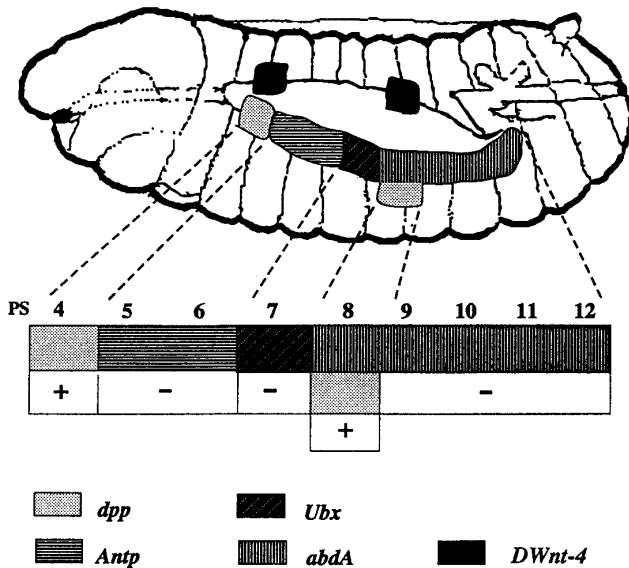


Fig. 7. Summary of *DWnt-4* regulation in the VM. The scheme depicts the expression patterns of *DWnt-4* (upper part) and of *dpp*, *Antp*, *Ubx* and *abd-A* (lower part) in the VM of a stylised stage 14 embryo. The approximate parasagittal positions of *dpp*, *Antp*, *Ubx* and *abd-A* expression, and the regulation, activation (+) or repression (-), they exert on *DWnt-4* are given in the linear projection shown beneath. *Antp*, *Ubx* and *abd-A* repress *DWnt-4* transcription in PS5-6, PS7 and PS9-12 respectively. *Ubx* and *abd-A*, which play dual roles in the control of *DWnt-4*, are also required for *DWnt-4* activation in PS8. *DWnt-4* activation in PS8 by *Ubx* is clearly indirect and presumably involves the *Dpp* signalling molecule. *Dpp* is also required for activation in PS4.

have been described: *Ubx* can substitute for *Abd-A*, and *Abd-A* for *Abd-B* in the down-regulation of *Dll* (Vachon et al., 1992) and *scabrous* (Graba et al., 1992) in abdominal segments. Analysing the repression of the *Antp* P2 promoter by *Ubx* and *Abd-A*, Appel and Sakonju (1993) proposed a model where inactivation results from a steric blockade due to the fixation of HOM-C gene products on homeodomain binding sites, rather than from specific interactions with trans-activating factor(s). The model provides a simple and attractive explanation as to the interchangeability of HOM-C proteins in the repression of a common target.

Ubx and *Abd-A* are required for *DWnt-4* expression in PS8. *Ubx* acts indirectly; it activates *dpp* in PS7 (Capovilla et al., 1994), which in turn stimulates *DWnt-4* in PS8. Two scenarios are possible regarding the role of *abd-A*. In the first, transcriptional activation would only require the *dpp* signal received from PS7, and *Abd-A* protein would have no direct and positive effect on *DWnt-4*. In *abd-A* mutants, the ectopic accumulation of *Ubx* would be sufficient to account for the silencing seen in PS8. Thus, the function of *abd-A* would serve only to prevent *Ubx* expression posterior to PS7. Alternatively, *DWnt-4* transcription in PS8 depends on the coordinate action of *Dpp* and *Abd-A*, as does *wg*. In that case, the apparent paradox that *Abd-A* acts as both an activator (PS8) and a repressor (PS9-12) is not resolved, which could suggest that a co-factor determines the specificity of action of *Abd-A*. For example, interaction of *Abd-A* with a PS8-specific protein

could be required for the activation of *DWnt-4*. The absence of the co-factor in more posterior PS would then lead *Abd-A* to act as a repressor. Growing evidence for the existence of co-factors for homeodomain proteins is currently emerging (Manak and Scott, 1993).

Ubx and *abd-A* control gut morphogenesis by driving the expression of secreted signalling factors in two adjacent domains at the boundary of which the second midgut constriction will form. The functions of *Dpp* and *Wg* in this morphogenetic event have been demonstrated (Bienz, 1994). The data reported in this paper indicate that a third signalling molecule, *DWnt-4*, whose expression in PS7-8 depends on *Ubx* and *abd-A*, could also be involved in this process. The isolation of mutants deficient for *DWnt-4* in the VM is a requisite for addressing this point.

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