DEVELOPMENTAL BIOLOGY 168, 202-213 (1995)

The *Drosophila* Wnt Protein DWnt-3 Is a Secreted Glycoprotein Localized on the Axon Tracts of the Embryonic CNS

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Accepted December 2, 1994

The *Wnt* gene family encodes highly conserved cysteine-rich proteins which appear to act as secreted developmental signals. Both the mouse *Wnt-1* gene and the *Drosophila wingless* (*wg*) gene play important roles in central nervous system (CNS) development. *wg* is also required earlier, in the development of the embryonic metameric body pattern. We have begun to characterize the developmental expression and role of another member of the *Drosophila Wnt* gene family, D*Wnt-3*. Using antisera raised to the DWnt-3 protein, we show that the protein is secreted *in vivo*. The early protein expression domains include the limb and appendage primordia. Late expression domains comprise the ventral cord and supraesophageal ganglia of the CNS. Notably, DWnt-3 protein accumulates on the commissural and longitudinal axon tracts of the CNS. Ectopic expression of D*Wnt-3* in transgenic embryos bearing a HS-D*Wnt-3* construct leads to specific disruption of the commissural axon tracts of the CNS. We also show that D*Wnt-3* does not functionally replace *wg* in an *in vivo* assay. Experiments with a tissue culture cell line transfected with a construct encoding the D*Wnt-3* gene show that DWnt-3 protein is efficiently synthesized, glycosylated, proteolytically processed, and transported to the extracellular matrix and medium. D*Wnt-3*, therefore, encodes a secreted protein, which is likely to play a role in development of the *Drosophila* CNS.

INTRODUCTION

The expression patterns of many members of the *Wnt* gene family suggest that they have functions in the development of the central nervous system (CNS) (reviewed in Nusse and Varmus, 1992). For example, *Wnt-1* is required for development of the CNS, as inferred from the phenotypes of embryos lacking the *Wnt-1* gene product (McMahon and Bradley, 1990; Thomas and Capecchi, 1990). Homozygous mutant embryos display varying degrees of disruption of cerebellar and midbrain structure. The mouse *Wnt-3A* gene is also involved in CNS development (Takada *et al.*, 1994), but the roles of other *Wnt* genes in CNS development have not yet been evaluated.

The discovery that the *Drosophila* segment polarity gene, *wingless* (*wg*) is a member of the *Wnt* gene family (Cabrera *et al.*, 1987; Rijsewijk *et al.*, 1987) has allowed utilization of the developmental genetics of the fruit fly to analyze Wnt protein function. Much of the work concerning *wg* has focused upon its role in the specification of segment polarity during early embryonic development (reviewed in Perrimon, 1994). Recent studies have indicated a role for *wg* in the development of the *Drosophila* embryonic and adult CNS (Patel *et al.*, 1989; Chu-Lagraff and Doe, 1993; Kaphingst and Kunes, 1994).

The *Drosophila* embryonic CNS originates from a sheet of cells comprising the ventral neuroectoderm (reviewed in Goodman and Doe, 1993). In each hemisegment, 25 neuronal precursor cells, called neuroblasts, delaminate and give rise to motoneurons, interneurons, and neurosecretory neurons. Many neurons eventually extend processes across the ventral midline via a mechanism requiring interactions with glial cells at or near the midline. Axons fasciculated together travel parallel to each side of the midline and across the midline, resulting in a characteristic "ladder-like" appearance of the embryonic CNS.

wg is expressed in the neuroectoderm and a subset of the neuroblasts in each hemisegment of the CNS (Doe, 1992; Chu-Lagraff and Doe, 1993). Lack of functional Wg protein results in the failure of the RP2 motoneuron to develop (Patel *et al.*, 1989; Chu-Lagraff and Doe, 1993). This motoneuron arises from neuroblasts that do not express *wg* themselves, but are born adjacent to the *wg*-expressing neuroectoderm (Doe, 1992). Similarly, *wg* is non-cell-autonomous in its action during segmentation; it is required in cells adjacent to the *wg*-transcribing

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0012-1606/95 $6.00

cells (Morata and Lawrence, 1977; Baker, 1988). These observations are consistent with the hypothesis that *wg* acts as a secreted factor.

Common structural features of *Wnt* gene products further indicate that they act as extracellular signalling molecules. Wnt proteins bear one or more potential N-linked glycosylation sites, a potential amino-terminal signal sequence and a framework of approximately 22 cysteines whose relative spacing is conserved (reviewed in Nusse and Varmus, 1992). Both the Wnt-1 (Papkoff, 1989; Bradley and Brown, 1990) and wg (van den Heuvel *et al.*, 1993) proteins are secreted from tissue culture cells transfected with expression constructs. Immunochemical evidence for *in vivo* secretion exists only for the Wg protein (van den Heuvel *et al.*, 1989; González *et al.*, 1991), presumably due to the difficulty in raising antisera that recognize the native structures of Wnt proteins. *wg* is secreted from the cells in which the gene is transcribed and remains largely associated with the extracellular surface of those cells. Wg protein can also be detected in multivesicular bodies in the adjacent *en*-expressing cells. Cell-free medium derived from cultures of *wg*-producing cells displays an activity, inhibited by anti-*wg* antisera, in a tissue culture assay measuring stabilization of the Armadillo protein (Van Leeuwen *et al.*, 1994), a known downstream effect of the *wg* signal in the embryo (Riggleman *et al.*, 1990).

To further our understanding of Wnt protein action in development and to begin to address aspects of the specificity of *Wnt* gene family members *in vivo*, we previously cloned two additional members of the *Drosophila Wnt* gene family, D*Wnt-2* and D*Wnt-3* (Russell *et al.*, 1992). The latter has also been named D*Wnt-5* (Eisenberg *et al.*, 1992). A fourth *Drosophila Wnt* gene, *Sternopleural* (*Sp*), has recently been cloned as a downstream target of the *Ubx* homeotic gene (Graba *et al.*, 1995).

D*Wnt-2* and D*Wnt-3* are expressed in specific and dynamic patterns during embryonic development as revealed by RNA *in situ* analysis (Eisenberg *et al.*, 1992; Russell *et al.*, 1992). Both D*Wnt-2* and D*Wnt-3* are transcribed in the presumptive limb primordia. D*Wnt-3* transcripts are also found in the labrum and antennal region and in patches of cells in the gnathal segments. Expression of the D*Wnt-3* gene during late embryogenesis is predominantly localized to the CNS.

In this paper, we present an analysis of the *in vivo* expression domains of the D*Wnt-3* protein and properties of D*Wnt-3* protein produced by tissue culture cells, using region-specific antisera. We also show that ubiquitous expression of the D*Wnt-3* gene driven by a heat-shock promoter in transgenic embryos results in severe disruption of the CNS commissural axon tracts. The expression domains of the D*Wnt-3* protein and the consequences of its ectopic expression suggest that D*Wnt-3* plays a role in the development of the embryonic CNS.

RESULTS

Early Embryonic Expression Domains of the DWnt-3 Protein

To localize DWnt-3 protein in whole-mount embryos, we raised antisera in rabbits against trpE-fusion proteins containing unique regions of D*Wnt-3*. The location of these regions in the DWnt-3 protein is shown (Fig. 1). Polyclonal antisera raised against both regions recognize denatured D*Wnt-3* on Western blots (see Results below). Anti-Region A antiserum also recognizes DWnt-3 protein in whole-mount embryos. The specificity of the antiserum was confirmed by the absence of staining in embryos homozygous for the deficiency, Df(1) N19 (Craymer and Roy, 1980; data not shown), which uncovers the genomic region to which D*Wnt-3* was localized by *in situ* hybridization of polytene chromosomes (Eisenberg *et al.*, 1992; Russell *et al.*, 1992). DWnt-3 protein is found in a pattern similar to that established previously by RNA *in situ* analysis (Eisenberg *et al.*, 1992; Russell *et al.*, 1992). At stage 10, immunoreactive cells are present in bilaterally symmetrical regions corresponding to the labrum and antennal primordia and the labial and maxillary gnathal segments (Figs. 2A and 2C). Inspection at higher magnification reveals that DWnt-3 protein is apparently secreted and spreads adjacently (Fig. 2B). At stage 11, DWnt-3 protein is found in ventral patches of cells corresponding to the thoracic limb primordia (Fig. 2D). DWnt-3 protein is predominantly localized at or adjacent to the surface of cells transcribing the D*Wnt-3* gene (Fig. 2B).

Expression of DWnt-3 Protein in the Developing CNS

D*Wnt-3* transcripts were previously found in the supraesophageal ganglia of the brain and in the ventral nerve cord (Russell *et al.*, 1992). Immunostaining of whole-mount embryos with the anti-D*Wnt-3* Region A antiserum reveals that DWnt-3 protein is found in each neuromere at stage 11 (data not shown). As the germ band retracts and the neuromeres of the gnathal segment condense to form the subesophageal ganglia, protein expression also becomes evident in the supraesophageal ganglia of the brain (Fig. 3C). At stage 13, DWnt-3 protein is seen throughout the ventral nerve cord at a low level; strong immunoreactivity is apparent in each hemisegment in a pattern suggestive of the commissural and longitudinal axon bundles (Fig. 3B).

The apparent localization of DWnt-3 protein to the axonal bundles of the ventral cord may reflect the transcription of D*Wnt-3* in the neuronal cell nuclei with subsequent translation and transport of DWnt-3 protein to the axonal processes. Intense staining might be apparent only in areas of high axon density, i.e., the commissural and longitudinal axon tracts. Another possibility

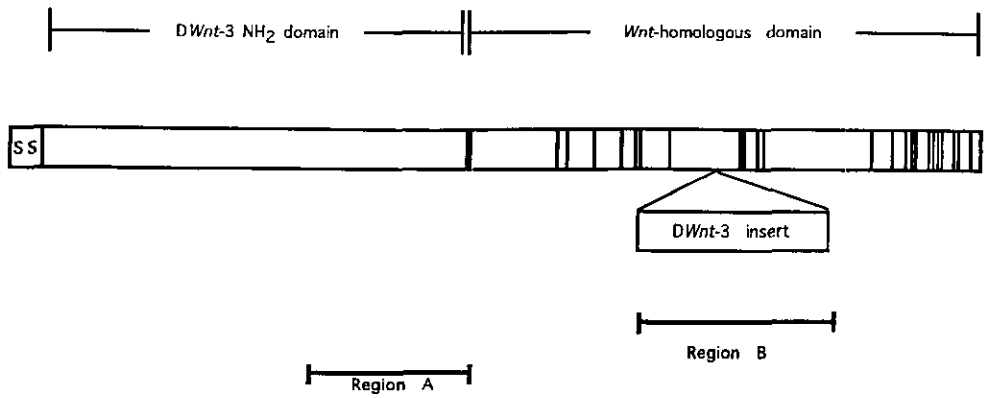

FIG. 1. Schematic representation of the DWnt-3 coding region and location of the regions included in fusion proteins. The Wnt-homologous domain and the unique DWnt-3 regions (the NH_2 domain and the insert) are indicated. ss denotes the putative signal peptide, the thin vertical bars represent cysteine residues, and the thick vertical bar marks the border between the Wnt-homologous domain and the unique amino-terminal domain. The region A fragment has endpoints at the D*Wnt-3* *Cla*I sites at nucleotides 1191 and 1753. The region B fragment has endpoints at the *Cla*I and *Nar*I sites at nucleotides 2147 and 2807, respectively.

is that the DWnt-3 protein is produced and secreted by accessory glial cells underlying the axon tracts and subsequently accumulates on the surface of the axonal processes. To attempt to distinguish between these two possibilities, we have compared expression patterns of DWnt-3 protein and RNA with the previously characterized expression patterns of antigens recognized by two monoclonal antibodies (MAb), MAbBP102 and MAb44C11.

MAbBP102 recognizes an antigen present on the cell surface of all neuronal cells (C. Goodman *et al.*, personal communication). Comparison of the expression patterns of DWnt-3 protein in the ventral nerve cord (Fig. 3B) and in the brain (Fig. 3C) with those of the antigen recognized by MAbBP102 (Figs. 3E and 3F), indicate that DWnt-3 protein is found on a subset of the cells positive for the MAbBP102 antigen. The MabBP102 antigen is also found on the neuronal cells of the peripheral nervous system (PNS); we have not found evidence for expression of DWnt-3 protein in the PNS.

RNA *in situ* analysis using a D*Wnt-3* probe shows that D*Wnt-3* RNA is found uniformly in the presumptive neuronal cells of the brain (data not shown) and ventral nerve cord (Fig. 3A). Confirmation that the cells transcribing D*Wnt-3* are indeed neuronal, comes from the comparison of the D*Wnt-3* transcription expression patterns with whole-mount immunostainings using MAb44C11. This monoclonal antibody recognizes nuclei throughout the ventral nerve cord and brain (Bier *et al.*, 1988; Fig. 3D and data not shown) which appear identical to those which transcribe the D*Wnt-3* gene. PNS neurons are also detected with MAb44C11, which do not express D*Wnt-3* RNA (Fig. 3A).

Further evidence of the transcription of D*Wnt-3* throughout the ventral cord was provided by the dissection of whole mount embryos used for *in situ* hybridization with a D*Wnt-3* probe. The ventral cord can be removed as an intact discrete tissue; examination of the dissected ventral cord indicates that the border of D*Wnt-3*-transcribing tissue is coincident with that of the ventral cord (data not shown). While we have not excluded the possibility that some glial cells secrete DWnt-3 protein which accumulates upon the axon tracts, our data are most consistent with the transcription of D*Wnt-3* in a majority of ventral nerve cord neuronal cell nuclei and subsequent translation and transport of DWnt-3 protein to the neuronal cell processes.

Localization of DWnt-3 to a Region on the X Chromosome Defined by Several Deficiencies

To begin a genetic dissection of the role of D*Wnt-3* in embryonic development, we have examined a series of mutant *Drosophila* strains carrying deficiencies in the region where D*Wnt-3* is localized by *in situ* hybridization to polytene chromosomes, 17A-B (Eisenberg *et al.*, 1992; Russell *et al.*, 1992). Embryos homozygous for the deficiency were examined for DWnt-3 staining in either the gnathal segments or the CNS. The lack of DWnt-3 protein in a subset of the homozygous deficiency strain embryos tested (Table 1) shows that D*Wnt-3* is located between 17B3 and 17C2, in good agreement with the polytene chromosome *in situ* hybridizations.

DWnt-3 Does Not Functionally Replace wg in Vivo

Our previous work demonstrated that ubiquitous *wg* expression results in the generation of an ectopic *en* expression domain and larvae with a naked cuticle ((Noordermeer *et al.*, 1992); Figs. 4C and 4F). To address whether D*Wnt-3* and *wg* can interact with a common downstream pathway during segmentation, we tested the ability of the

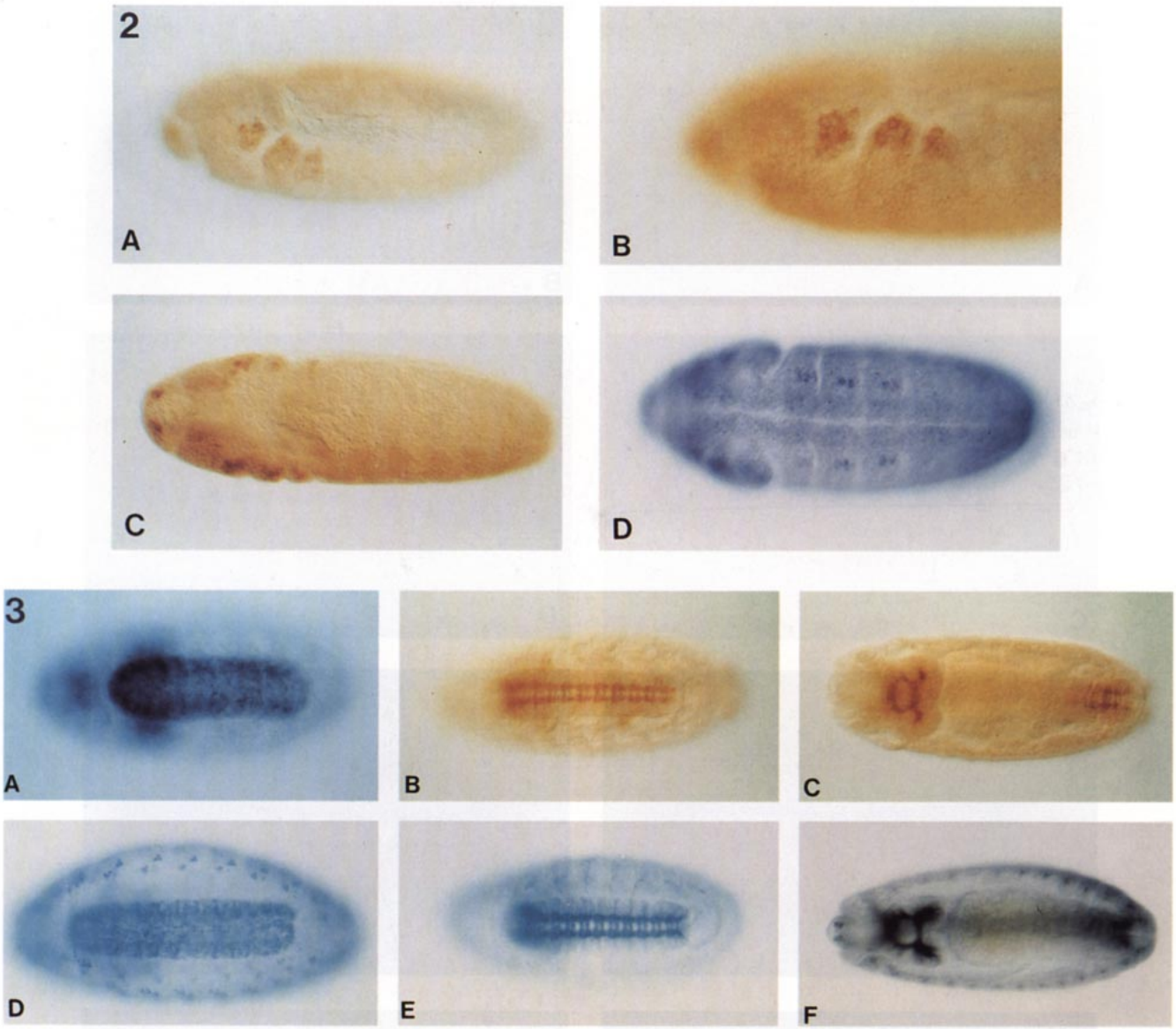

FIG. 2. Early expression domains of the DWnt-3 protein. The expression patterns of DWnt-3 protein in stage 10/11 wildtype embryos are shown. (A) Lateral surface view showing DWnt-3 protein expression in the gnathal segments. (B) Close up of the embryo shown in A. (C) Ventral sagittal view showing staining in the labrum. (D) Ventral surface view showing staining in the thoracic limb primordia. Anterior is to the left in all photographs. Dorsal is up in A and B.

FIG. 3. DWnt-3 protein expression domains in the CNS. The expression patterns of D*Wnt-3* RNA, DWnt-3 protein, and the MAb-BP102 and MAb-44C11 epitopes in the wildtype embryonic CNS are shown. (A) Ventral view of a whole-mount RNA *in situ* using a D*Wnt-3* cDNA probe showing staining throughout the ventral nerve cord. (B) Ventral view showing the expression pattern of DWnt-3 protein in the nerve cord. (C) Sagittal view showing DWnt-3 protein expression in the supraesophageal ganglia of the brain. (D) Ventral view showing the CNS and PNS immunostaining pattern seen with Mab-44C11. (E) Ventral view showing the CNS and PNS immunostaining pattern seen with Mab-BP102. (F) Sagittal view showing brain ganglia detected with MAb-BP102.

uniform D*Wnt-3* expression in HS-D*Wnt-3* embryos to alter *en* expression and cuticle morphology.

Three transgenic lines bearing independent insertions of a P-element transposon with D*Wnt-3* under HS promoter control, marked with the *white*$^+$ gene, were established and tested. Heatshock of the HS-D*Wnt-3* transgenic embryos leads to high uniform expression of DWnt-3 protein as revealed by whole-mount immunostaining (Fig. 5B). Ubiquitous expression of D*Wnt-3*, during the same developmental stages where ubiquitous *wg* expression in HS-*wg* embryos has the described effect, results in essentially wildtype cuticle morphology and *en* expression domains (Figs. 4B and E). Thus, D*Wnt-3* and *wg* are not functionally equivalent during segmentation, suggesting that D*Wnt-3* does not productively interact with the *wg* reception pathway. Exam-

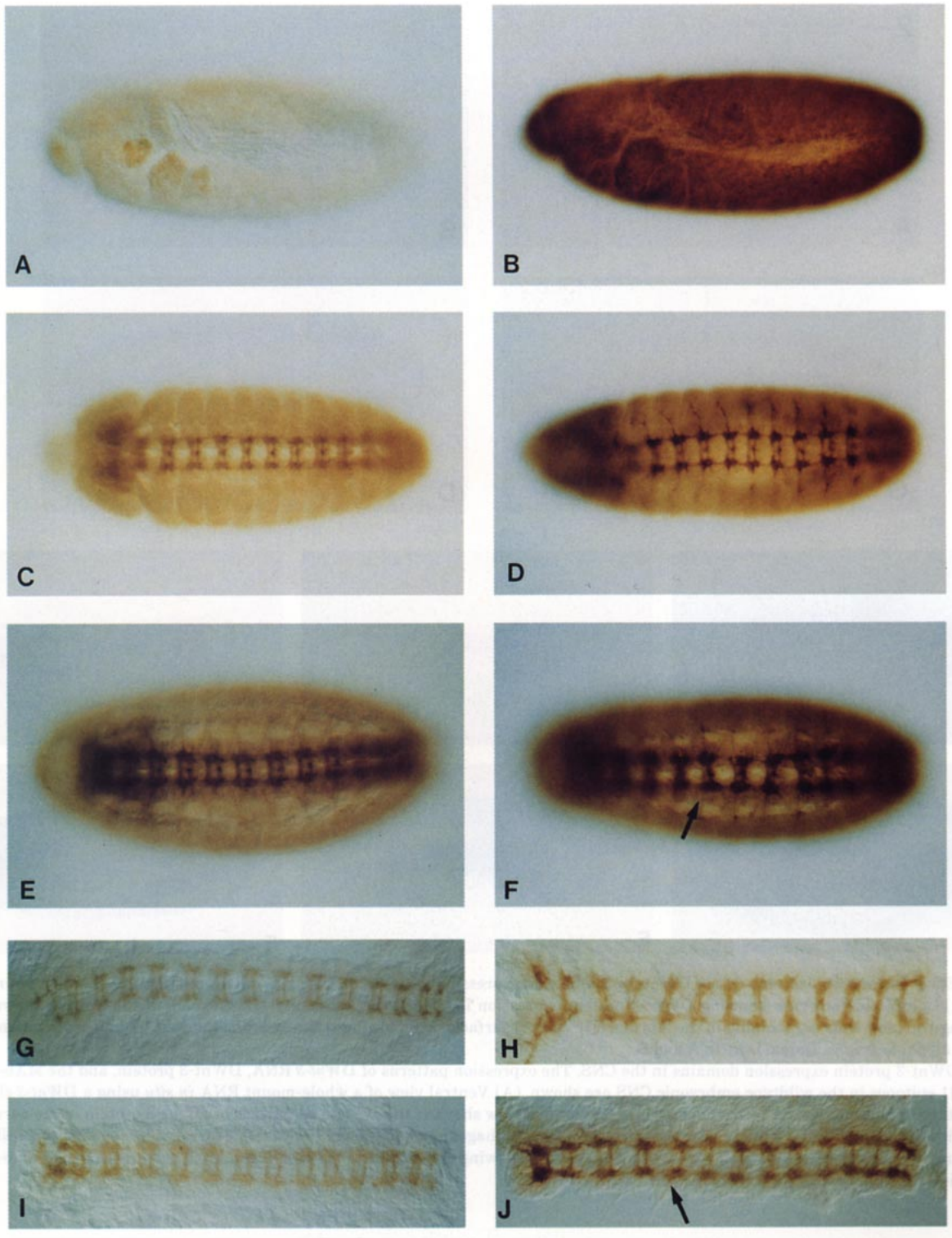

FIG. 5. Ectopic DWnt-3 expression leads to abnormalities in the axon tracts of the embryonic CNS. DWnt-3 protein expression and the axon tracts of the CNS detected with Mab-BP102 are shown. Embryos shown in A and B are immunostained with the anti-DWnt-3 Region B antisera; those in C–J are immunostained with MAb-BP102. A and B are lateral surface views and C–F are ventral surface views of whole-mount embryos. G–J are dissections of ventral nerve cord and associated body wall tissue. (A) DWnt-3 protein expression in a whole-mount stage 10 wildtype embryo. (B) Ubiquitous DWnt-3 protein expression in a whole-mount heatshocked stage 10 HS-D*Wnt-3* embryo. (C) Stage 13 wildtype embryo. (D) Stage 13 HS-D*Wnt-3* embryo. (E) Stage 15 wildtype embryo. (F) Stage 15 HS-D*Wnt-3* embryo (arrow indicates abnormal commissures). (G) Stage 13 wildtype embryo. (H) Stage 13 HS-D*Wnt-3* embryo. (I) Stage 15 wildtype embryo. (J) Stage 15 HS-D*Wnt-3* embryo (arrow indicates abnormal commissures). In all panels, anterior is to the left, with dorsal up in A and B.

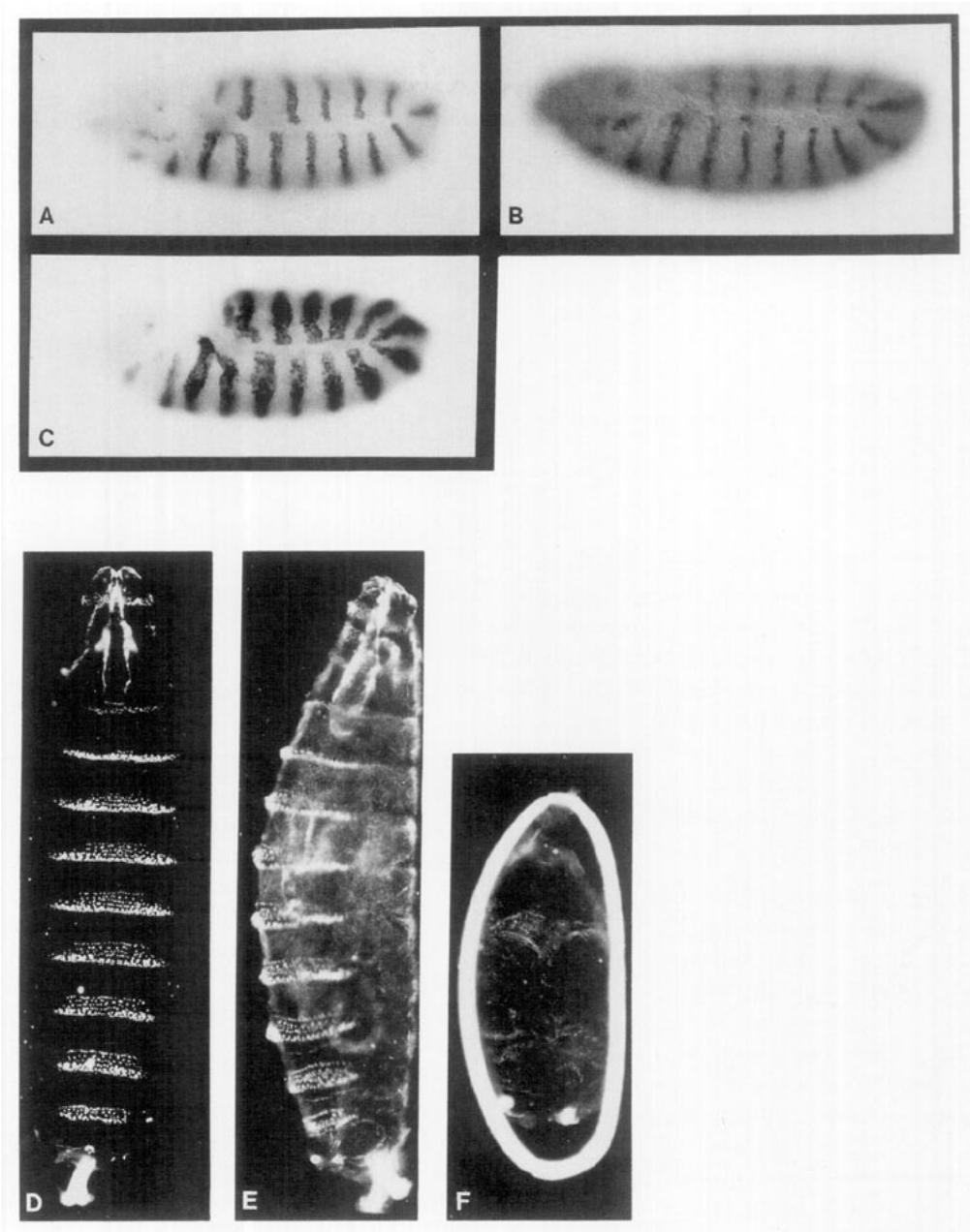

FIG. 4. D*Wnt-3* does not functionally replace *wg in vivo*. The En protein expression domains and larval cuticle patterns in wildtype, HS-D*Wnt-3* and HS-*wg* embryos are shown. All embryos were heatshocked as described under Materials and Methods. (A) En expression in wildtype embryo. (B) En expression in the HS-D*Wnt-3* embryo resembles the wildtype En pattern. (C) En expression in HS-*wg* embryo showing expansion of the En expression domain. Anterior is to the left, dorsal is up. (D) Cuticle pattern of a wildtype larvae. (E) Cuticle pattern of HS-D*Wnt-3* resembling that of the wildtype larvae. (F) Cuticle pattern of the HS-*wg* embryo showing the naked phenotype.

ination of the regions of the embryo where D*Wnt-3* is normally expressed, however, revealed alterations in the CNS as discussed below.

Ectopic Expression of DWnt-3 Results in Disruption of the CNS

To analyze the consequences of misexpression of DWnt-3 in the CNS, we examined heatshocked HS-D*Wnt-3* embryos which were allowed to develop until the CNS is normally fully formed, by immunostaining with the BP102 antibody. Heatshock of D*Wnt-3* transgenic embryos resulted in significant malformation of commissural axon tracts (Figs. 5D and 5F). The abnormally thin width of the commissures is presumably due to a reduction in axon bundles crossing the ventral midline. Simultaneous heatshocks of identically treated wildtype embryos (Figs. 5C and 5E) or HS-*wg*

TABLE 1
ANTI-D*Wnt-3* STAINING OF X CHROMOSOME DEFICIENCY EMBRYOS

Deficiency	Cytology[a]	D*Wnt-3* staining[b]	Reference[c]
Df(1)N19	17A3-6; 17F2-18A3	−	2
Df(1)os^{UE19}	17A5; 17A12	+	4
Df(1)D2	17A7-9; 17B2	+	4
Df(1)fu^{H4}	17C3; 17D2	+	1
Df(1)fu^{B10}	17C5-D1; 18A4-7	+	1
Df(1)E128	17B3-C2; 18A-B	−	3
Df(1)fu^{E5}	17B3-C1; 17E7-F3	−	1

[a] Cytology reported in Eberl *et al.* (1992).
[b] Homozygous deficiency-bearing embryos were scored for DWnt-3 expression by whole-mount immunostaining.
[c] 1, Busson *et al.* 1988; 2, Craymer and Roy, 1980; 3, Engels and Preston, 1981, and 4, Eberl *et al.* (1992).

embryos (data not shown) did not result in any apparent disruption of the CNS. Therefore, ectopic expression of D*Wnt-3* during the stages where it is normally found on the ventral cord commissural and longitudinal axon tracts interferes with the proper formation of the commissural axon tracts.

Synthesis and Processing of the DWnt-3 Protein Expressed in an Imaginal Disc Cell Line

To further our understanding of the biosynthesis of the DWnt-3 protein, we wished to express the gene in a tissue culture cell line which normally expresses the protein, making it likely that any post translational modifications observed are relevant *in vivo.* Northern blot analysis of RNA from two imaginal disc cell lines (Peel and Milner, 1989), Clone 8, derived from the wing disc, and L-127, derived from a leg disc, showed that the D*Wnt-3* gene is transcribed in these cells (data not shown). We characterized DWnt-3 protein produced by untransfected Clone 8 cells and Clone 8 cells stably transfected with a D*Wnt-3* expressing construct. Cells were transfected with expression constructs bearing the D*Wnt-3* gene in both the sense and antisense orientations under heatshock promoter control and polyclonal cell lines were derived. Analysis of DWnt-3 protein present in untransfected cells by Western blot analysis using the anti-D*Wnt-3* Region A antiserum reveals a polypeptide of ~140 kDa (Fig. 6A, lane 1). Heat shock treatment of cells bearing the antisense orientation of the expression construct leads to a decrease in the amount of 140 kD species (Figure 6A, lane 2), suggesting that the 140-kDa species is encoded by the D*Wnt-3* gene. Heatshock treatment of cells bearing the sense orientation of the expression construct leads to an increase in the 140-kDa species and a species migrating at 80 kDa becomes evident (Fig. 6A, lane 3).

The molecular weight of D*Wnt-3* predicted from the primary amino acid sequence of the polypeptide encoded by the D*Wnt-3* gene is 113 kDa (Eisenberg *et al.*, 1992; Russell *et al.*, 1992). To investigate the nature of post-translational modifications that might account for the apparent discrepancy between the predicted and observed sizes of the DWnt-3 protein, we tested the possibility that one or more of the 13 potential N-linked glycosylation sites in the predicted amino acid sequence of D*Wnt-3* is utilized during its synthesis.

Cells bearing the sense orientation of the expression construct were heatshocked during a metabolic labeling in the presence or absence of tunicamycin, an inhibitor of N-linked glycosylation. Treatment with tunicamycin led to a decrease in the 140-kDa species with a commensurate increase in a species which migrates at ~116 kDa (Fig. 6B), in good agreement with the predicted molecular weight of the DWnt-3 protein. This result suggests that N-linked glycosylation accounts for much, if not all, of the post-translational modification of D*Wnt-3* detectable by SDS–PAGE.

Secreted DWnt-3 Is Proteolytically Processed and Found in the Extracellular Matrix and the Medium

To examine the possible secretion of the DWnt-3 protein, cells bearing the heatshock D*Wnt-3* expression constructs were either left untreated or heatshocked in serum-free media. Protein extracts were prepared from the extracellular matrix fraction, the sedimentable media component, the postsedimentation media supernatant, and cells. The extracellular matrix fraction is operationally defined as that material left on the plastic tissue culture dish surface after mechanical removal of the adherent cells and the washing away of remaining soluble proteins.

Upon heatshock treatment, the 140-kDa species and the 80-kDa species of D*Wnt-3* are found in all cellular and extracellular fractions (Figs. 7A and 7B). Both species are recognized by both the anti-D*Wnt-3* Region A and anti-D*Wnt-3* Region B antisera. While small amounts of the 140-kDa DWnt-3 species are found in the postsedimentation media supernatant, the 80-kDa species is the predominant immunoreactive form (Figs. 7A and 7B). In untransfected Clone 8 cells, DWnt-3 protein is detected only in the extracellular matrix fraction and not in the medium in any form (data not shown). Therefore, D*Wnt-3* is efficiently synthesized, glycosylated, proteolytically processed to yield a form bearing the conserved *Wnt*-homologous domain, and secreted to the extracellular matrix and to the medium in both sedimentable and nonsedimentable forms by the tissue culture cells.

DISCUSSION

Evidence presented in this paper indicates that the *Drosophila* DWnt-3 protein is expressed during embry-

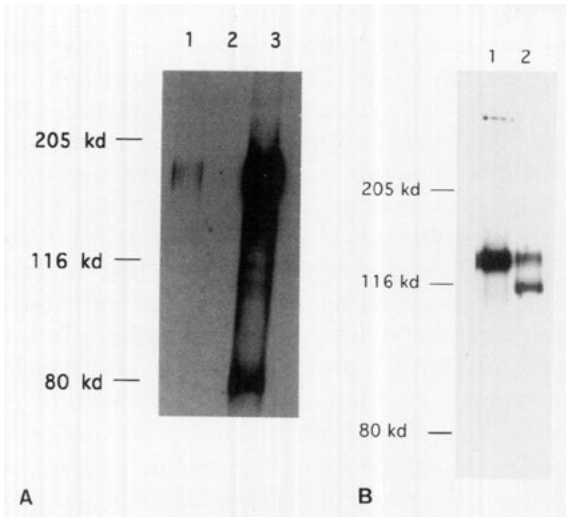

FIG. 6. DWnt-3 protein produced by an imaginal disc cell line is glycosylated. (A) Whole cell extracts were prepared from heatshocked untransfected and HS-D*Wnt-3*-transfected imaginal disc cell lines. DWnt-3 protein was detected by Western blot analysis using the anti-Region B antiserum. Lane 1 contains extract prepared from untransfected cells, lane 2, HS-D*Wnt-3* (antisense orientation)-transfected cells and lane 3 HS-D*Wnt-3* (sense orientation)-transfected cells. (B) The HS-D*Wnt-3* (sense orientation)-transfected cell line was metabolically labeled with [^{35}S]cysteine and the DWnt-3 protein was subsequently immunoprecipitated. Lane 1 contains polypeptides immunoprecipitated from untreated cells, lane 2, from cells treated with tunicamycin during the metabolic labeling. The molecular weights of marker proteins are indicated to the left of each panel.

ogenesis in the labrum, presumptive primordia of antennal and limb regions, patches of cells in the labial and maxillary gnathal segments, the brain ganglia, and the commissural and longitudinal axon tracts of the CNS.

The D*Wnt-3* gene is transcribed in cells of the ventral neurogenic region beginning at stage 11 (Russell *et al.*, 1992), the stage at which the newborn neuroblasts delaminate from the neuroectodermal sheet (reviewed in Goodman and Doe, 1993). We have shown in this work that, during later developmental stages, D*Wnt-3* mRNA is found in the field of neuronal cell nuclei which fill the ventral nerve cord. The protein is found on the commissural and longitudinal axon tracts, presumably, due to its display on the surface of the axonal process and the density of processes in those areas. Although we know little about downstream target cells of the D*Wnt-3* signal, it is possible that they may be accessory cells in the CNS in the immediate environment of the axon bundles, such as glial cells.

Midline glial cells are required for proper formation of the ventral cord commissures of the CNS (reviewed in Goodman and Doe, 1993). Extracellular factors secreted by these cells, such as the *slit* protein (Rothberg *et al.*, 1990), have been shown to have important roles in the interaction between the glia and pioneering neuronal axons. Conversely, DWnt-3 protein displayed on the neuronal processes may play a role in neuron-glial cell interactions, i.e., in the proliferation, development, or maintenance of glial cells. While this has not yet been demonstrated in the fruitfly, mammalian neurons have been shown to secrete neurohormones and neurotransmitters that affect functions of the astrocyte and oligodendrocyte glial lineages (reviewed in Vernadakis, 1988). DWnt-3 protein displayed on the neuronal process may also have an autocrine role in particular aspects of neuronal function.

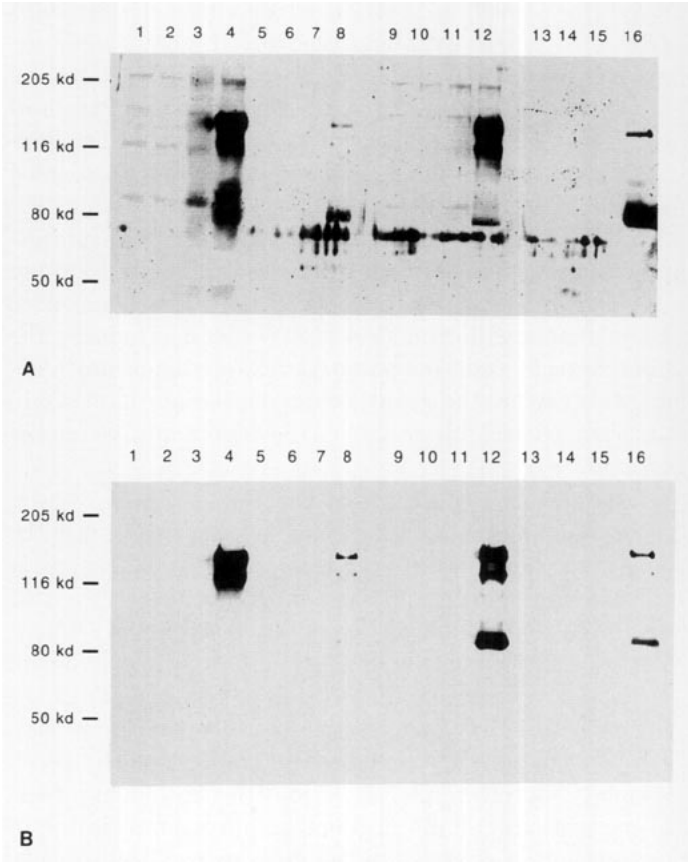

FIG. 7. DWnt-3 is proteolytically cleaved and secreted to the extracellular matrix and the media. The imaginal disc cell lines bearing the HS-D*Wnt-3* construct in either the sense or antisense orientation were left untreated or heatshocked and whole cell extract, the sedimentable media component, extracellular matrix fraction, and the postsedimentation media supernatant were prepared and analyzed by Western blot. The molecular weights of marker proteins are indicated to the left of each panel. (A) The primary antiserum used for detection of the DWnt-3 protein was raised against the Region A fusion protein. For lanes 1, 5, 9, and 13, protein was derived from untreated HS-D*Wnt-3* (antisense orientation) cells; for lanes 2, 6, 10, and 14, protein was derived from heatshocked D*Wnt-3* (antisense orientation) cells; for lanes 3, 7, 11, and 15, protein was derived from untreated HS-D*Wnt-3* (sense orientation) cells, and for lanes 4, 8, 12, and 16 from heatshocked HS-D*Wnt-3* (sense orientation) cells. The whole cell extracts (lanes 1 through 4), sedimentable media component (lanes 5 through 8), extracellular matrix fraction (lanes 9–12), and the postsedimentation media supernatant (lanes 13 through 16) were analyzed. (B) The primary antiserum used for detection of the DWnt-3 protein was raised against the Region B fusion protein. The description of lanes is the same as that for A.

Evaluation of the role of D*Wnt-3* during development requires the eventual identification of a mutant strain of flies lacking a functional D*Wnt-3* gene. The region of the X chromosome to which D*Wnt-3* has been mapped, 17A-B, is well studied (Eberl *et al.*, 1992). We have shown that the D*Wnt-3* gene falls in an area defined by several deficiency chromosomes that is in good agreement with the original mapping by hybridization to polytene chromosomes. Two defined complementation groups fall in this area, *Beadex* (*Bx*) and *maggot* (*mgt*). Embryos derived from stocks of the 10 existing alleles of *mgt* and a strong *Bx* allele were analyzed for the embryonic expression of the DWnt-3 protein by immunostaining. All alleles tested displayed wildtype expression patterns of the DWnt-3 protein (data not shown). It remains possible that a mutant in the D*Wnt-3* gene may not display alteration of the embryonic expression patterns of the protein. It is also possible that another lethal complementation group in this interval remains to be found or that D*Wnt-3* is not an essential gene. We have not been able to assess the function of DWnt-3 by studying the phenotype of embryos homozygous for the various deficiencies around the gene, because the smallest ones still uncover several other known developmentally important genes.

Lacking a null mutant of the D*Wnt-3* gene, we exploited an approach used earlier to gain insight into the functions of a number of different gene products, namely the phenotypic consequences of ubiquitous expression driven by the heatshock promoter (reviewed in Gibson, 1991; see also Yang *et al.*, 1993). Ubiquitous expression of D*Wnt-3* in the developing CNS leads to defects in the formation of the commissural axon tracts. These commissures display DWnt-3 protein during normal development; therefore, the abnormalities may arise from overexpression of the protein, inappropriate timing of expression, or changes in the fates of a subset of cells, for example glial cells, which are required in the proper formation of the commissural axon tracts. Further studies will be necessary to distinguish among these possibilities. The effects of ubiquitous D*Wnt-3* protein expression seem specific to the CNS as no other abnormalities were seen.

We have demonstrated that the DWnt-3 protein is secreted both *in vivo* and *in vitro*. The protein is found in a cell line derived from dissociated wing imaginal discs. Increasing the level of expression of the DWnt-3 protein by heatshock treatment of cells stably transfected with the D*Wnt-3* gene under heatshock promoter control reveals that the protein is N-linked glycosylated, proteolytically cleaved, and secreted. Extracellular protein is found associated with the extracellular matrix and in the media. DWnt-3 protein is present in the media in both sedimentable and nonsedimentable forms.

The use of region-specific antisera reveals that the nonsedimentable D*Wnt-3* fraction is enriched for the species generated by proteolysis of the mature DWnt-3 protein. Apart from the inferred removal of their amino-terminal signal peptides, no specific proteolytic processing has been proposed or shown for the other Wnt proteins. Both species of secreted D*Wnt-3* contain part, if not all, of the highly conserved *Wnt*-homologous domain. The role and fate of the unique aminoterminal extension of the DWnt-3 protein is unclear. The scarce amounts of DWnt-3 protein in untransfected Clone 8 cells prevent evaluation of the relative amounts of proteolyzed product in these cells. It remains possible, therefore, that proteolysis does not usually efficiently occur and is the result of overproduction of the protein. Resolution of the question as to which species is active *in vivo* awaits development of an assay for D*Wnt-3* function.

Secreted DWnt-3 protein is partially retained by the extracellular matrix. Similar observations have been made for both the Wnt-1 and Wg proteins (Bradley and Brown, 1990; van den Heuvel *et al.*, 1993). DWnt-3 protein can be detected in the media of the heatshocked transfected cells, presumably because the large amounts of protein produced saturate the capacity of the ECM to bind DWnt-3 protein. While operationally defined as that extracellular material that is tightly associated with the cell and hence, difficult to correlate with functional structures *in vivo*, the extracellular matrix is the repository of a number of factors involved in intercellular signaling (reviewed in Juliano and Haskill, 1993). As has been shown for FGF (Klagsbrun and Baird, 1991; Rapraeger *et al.*, 1991; Yayon *et al.*, 1991), association of secreted factors with components of the extracellular matrix may be required for their interaction with high-affinity receptors. Conversely, association of factors with the extracellular matrix has also been postulated to limit their range of action and allows the regulation of their accessibility to their receptors. It may be necessary to retain Wnt proteins in the immediate vicinity of their site of synthesis. Indeed, ubiquitous expression of the Wg protein under heatshock promoter control in transgenic flies causes alterations in gene expression patterns that result in profound changes of cell identity throughout the segment (Noordermeer *et al.*, 1992).

The mechanisms for the specificity of Wnt protein unknown; the anticipated receptor(s) has not yet been identified. In this study, we have shown that ectopic expression of D*Wnt-3* in the HS-D*Wnt-3* transgenic embryo does not result in the broadening of the *en* expression domain or generation of ectopic naked cuticle, as seen with HS-*wg*. A tissue culture system has been identified which provides an assay of the activity of Wg protein provided by transfection of the cells with a *wg*-

expressing construct, coculture with *wg*-expressing cells or addition of cell free extracts (Van Leeuwen *et al.*, 1994). The assay measures the *wg*-dependent stabilization of the Armadillo protein, a known member of the *wg* signaling pathway in the embryo. DWnt-3 protein is not active in this assay when cells are transfected with the D*Wnt-3* expression construct (L.G.F., F. van Leeuwen, and R.N., unpublished), indicating that the reception pathways of *wg* and D*Wnt-3* do not overlap in these cells. Thus, both in a tissue culture system assay and *in vivo*, D*Wnt-3* does not functionally replace *wg*. The specificity of Wnt protein action *in vivo* may, therefore, be controlled both by the restriction of the expression domains of Wnt proteins and by the non-overlapping interactions between Wnt proteins and their reception pathways.

The expression patterns of D*Wnt-3* during embryogenesis and late larval development suggest that it is involved in the formation of several different tissue primordia. Perhaps most interesting is its potential involvement in the development of the CNS. The many molecular markers and mutant strains that have been generated in the study of the *Drosophila* CNS may prove useful in delineating the signaling pathway of D*Wnt-3*. Comparison of the various pathways through which different Wnt proteins act will further our understanding of their modes of action.

MATERIALS AND METHODS

Drosophila Stocks

The wildtype strain used was *CantonS-1*. Strains bearing deficiencies in the 17B region are listed in the legend to Table 1. Three independent HS-D*Wnt-3* lines were derived by P-element-mediated germline transformation of the pCaSpEr-HS-D*Wnt-3* construct described below. The HS-*wg* stock has been described previously (Noordermeer *et al.*, 1992).

Heatshock Procedure and Cuticle Preparations

To study the consequences of uniform DWnt-3 expression, embryos from three independent HS-D*Wnt-3* lines were heatshocked and their cuticle patterns and En expression patterns examined. In order to compare the possible effects of ubiquitous HS-D*Wnt-3* with the results of overexpression of Wg protein HS-*wg* embryos and *Canton-S* embryos were taken along as control. A heatshock regime was used that in HS-*wg* embryos results in a completely naked cuticle pattern and expansion of En expression as described in (Noordermeer *et al.*, 1992). For the study of the effects of uniform expression of DWnt-3 on CNS formation embryos were heatshocked using the same heatshock procedure, but embryos were now collected for 15 hr before heatshock treatment. For a detailed inspection of CNS structure the ventral cord of whole-mount embryos stained with MabBP102 was dissected with tungsten needles and mounted in 70% glycerol. Embryos were staged as described and larval cuticles were prepared according to standard procedures (Wieschaus and Nüsslein-Volhard, 1986).

Whole-Mount Antibody Stainings, RNA in Situ Hybridization

Whole-mount antibody stainings were performed as described (Noordermeer *et al.*, 1992) except that in some experiments, $NiCl_2$ was added to 0.3% final concentration during the HRP reaction, yielding a blue-colored precipitate as contrasted to the brown color of the DAB precipitate. RNA *in situ* hybridizations were performed as described (Noordermeer *et al.*, 1992).

Cell Lines and Transfections

The wing imaginal disc cell line, Clone 8, was obtained from D. Peel. The cells were grown in M3 media supplemented with insulin, fetal calf serum, and adult fly homogenate as described (Peel and Milner, 1989). The cells were transfected with the expression construct mixed at a 10:1 ratio with a plasmid bearing the hygromycin-resistance gene (Koelle *et al.*, 1991) using a standard $CaPO_4$ protocol. Cells were selected in growth media supplemented with 200 μg/ml hygromycin and pools of resistant cells were expanded and maintained in selective medium.

DNA Constructs

To express D*Wnt-3* under heatshock promoter control, a *Spe*I–*Eco*RV fragment containing the D*Wnt-3* gene and 3′ flanking sequences was blunt-ended and cloned into the *Stu*I site of pCaSpER-HS, a P-element construct bearing the HSP-70 promoter (C. Thummel, unpublished). Recombinant plasmids with insertions of the fragment in either orientation (sense and antisense) were identified and prepared by an alkaline lysis plasmid preparation protocol with the additional steps of a LiCl precipitation of high-molecular-weight RNA followed by RNase treatment and PEG precipitation. This construct was used to generate both the stably transfected tissue culture cell line and transgenic fly lines.

To express regions of D*Wnt-3* as trpE-fusion proteins, two fragments derived from the D*Wnt-3* cDNA were cloned into the appropriate PATH vector (Spindler *et al.*, 1984) to maintain the reading frame. The endpoints of these fragments are given in the legend of Fig. 1 (numbering according to Russell *et al.*, 1992). Construction of the fusion plasmid for Region A required propa-

gation of the D*Wnt-3* plasmid in a *dam-Escherichia coli* strain to allow cleavage at the *Cla*I site (nucleotide 1753) which has an overlapping *dam* methylation site.

A construct encoding a heterologous fusion protein bearing the domains present in both of the trpE-fusion protein constructs was made by cloning the *Spe*I–*Eco*RV fragment of the D*Wnt-3* gene (D*Wnt-3* has no introns) behind the phage T7 promoter in the pT7-7 plasmid (Tabor and Richardson, 1985).

Generation and Affinity Purification of Antisera

The recombinant plasmids encoding trpE-fusion proteins were introduced into the HB101 strain. Growth and induction of these cells was as described (Spindler *et al.*, 1984). The pT7-7 D*Wnt-3* construct was introduced into the XL1-Blue stain (Stratagene). Protein production was induced by infection of the cells with a M13 phage bearing the T7 polymerase gene under *lac* promoter control in the presence of IPTG (Tabor and Richardson, 1985). Inclusion bodies were prepared and fusion proteins purified by SDS–PAGE. Crushed polyacrylamide gel containing the fusion protein was used as an immunogen in rabbits. Immunopositive animals were identified by Western blot assay of their response to a heterologous fusion protein that does not contain a trpE-encoded region, pT7-D*Wnt-3*. Affinity purification of the D*Wnt-3* antisera greatly improved whole-mount immunostainings. Antisera were first negatively selected by passage over a column to which was attached inclusion body protein prepared from cells bearing the parental plasmid. The positive selection was done by passage over a column bearing the heterologous pT7-7 D*Wnt-3* fusion protein. Preparation of the column materials and chromatographic conditions were as described (Redding *et al.*, 1991).

Induction of Expression Constructs and Preparation of Extracts

Expression constructs were induced in the transfected tissue culture cells by a 30-min heatshock at 37°C in media lacking fetal calf serum and adult fly homogenate. After a 3-hr recovery at 25°C, cellular and extracellular fractions were prepared as follows.

Cell extracts. After two washes with PBS, cells were scraped into PBS and washed a further time in PBS. Material remaining in the tissue culture flask was used for preparation of the extracellular matrix fraction. Cells were lysed in NP-40 lysis buffer, nuclei spun out, and the resulting supernatant was mixed in equal volume with an SDS/urea-containing sample buffer prior to SDS–PAGE and Western blotting.

Extracellular matrix. After ensuring the lack of remaining adherent cells by microscopy, the SDS/urea-containing sample buffer was added directly to the flask which was heated to 100°C for 3 min by contact with a metal block in a boiling water bath.

Sedimentable and postsedimentation media components. Cell were removed by centrifugation and the supernatant was centrifuged for 90 min at 100,000*g* in a swinging bucket rotor. The pellet was solubilized by boiling in SDS/urea-containing sample buffer. The supernatant was concentrated approximately 10-fold by use of a Centricon-30 (Amicon) microconcentrating device, which was pretreated with 10 mg/ml of methylated BSA and washed with PBS prior to use. Equal volumes of the concentrated supernatant and the SDS/urea-containing sample buffer were mixed prior to boiling.

Metabolic Labeling, Tunicamycin Treatment, and Immunoprecipitations

Cell were plated to ca. 50% confluency in 12-well tissue culture dishes the evening prior to a heatshock induction which was performed as described above. [^{35}S]-Cystein (Amersham) was added (100 μCi/0.5 ml medium) in serum and adult-fly homogenate-free growth media lacking methionine and cysteine. Tunicamycin (Boehringer-Mannheim) was added to a concentration of 10 μg/ml, 30 min before addition of the radioactive medium. Cytoplasmic extracts were made as described above and DWnt-3 protein was immunoprecipitated by addition of crude anti-D*Wnt-3* Region B antiserum followed by capture of the immune complexes on Protein A–Sepharose beads (Pharmacia). The beads were washed three times with cold medium and the polypeptides were released from the matrix by boiling in the SDS/urea-containing sample buffer. The polyacrylamide gel resulting from SDS–PAGE was treated with Enhance fluor (Amersham) and radioactive polypeptides were visualized by autoradiography using an intensifying screen at −70°C.

We acknowledge C. Goodman, Y. N. Jan, and N. Patel for providing monoclonal antibodies, W. Engels, A. Hilliker, W. Mattox, and N. Perrimon for providing mutant *Drosophila* stocks, C. Thummel for the pCaSpER plasmid, M. Koelle for the hygroresistance plasmid, K. Redding for the antibody affinity purification protocol, and D. Peel for providing the imaginal disc cell lines. Thanks to Frank van Leeuwen for advice on the growth and transfection of Clone 8 cells, to Kathy Shaw for help in preparing the manuscript, and to members of the Nusse lab for their comments on the paper. L.G.F. thanks Bill Mattox and Norbert Perrimon for their helpful discussions. R.N. is an investigator of the Howard Hughes Medical Institute.

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