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ORIGINAL ARTICLE

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Analysis of the expression pattern of *Mysidium columbiae wingless* provides evidence for conserved mesodermal and retinal patterning processes among insects and crustaceans

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Abstract The Wnt family includes a number of genes, such as *wingless* (*wg*), which encode secreted glycoproteins that function in numerous developmental patterning processes. In order to gain a better understanding of crustacean pattern formation, a *wg* orthologue was cloned from the malacostracan crustacean *Mysidium columbiae* (mysid), and the expression pattern of this gene was compared with that of *Drosophila wg*. Although *Drosophila wg* is expressed in many developing tissues, such as the ventral neuroectoderm, *M. columbiae wg (mcowg)* expression is detected within only a subset of these tissues. *mcowg* is expressed in the dorsal part of each developing segment and within the developing eye, but not within the ventral neuroectoderm. Dorsal *wg* expression in *Drosophila* is required for heart and muscle development, and conservation of this dorsal *wg* expression pattern suggests that *mcowg* may function to pattern these tissues in mysids. Consistent with this, expression of Even-skipped (Eve) protein in heart precursor and muscle cells, which is dependent on Wg signaling in *Drosophila*, is also conserved in mysids. Within the developing mysid eye, *mcowg* is expressed in a pattern that is similar to the expression pattern of *Drosophila wg* in the fly eye disc. In *Drosophila*, Wg inhibits neural differentiation at the anterior margin of the eye disc and patterns the dorsal/ventral axis of the eye. These data indicate that *mcowg* may function similarly during mysid eye development. Analysis of *mcowg* expression provides molecular evidence suggesting that the processes of heart, muscle, and eye patterning are likely to be conserved among insects and crustaceans.

Keywords Wingless · Crustacean · Heart development · Muscle development · Eye development

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Introduction

The Wnt family includes a number of secreted glycoproteins that function as signaling molecules in numerous developmental processes. For example, the *Drosophila wg* gene, a *Wnt* family member, is required for the proper patterning of many tissues during fly development, including the epidermal cells that secrete the larval cuticle (Baker 1987, 1988; Dierick and Bejsovec 1999; Nusslein-Volhard and Wieschaus 1984). *wg* mutants secrete larval cuticles that lack regions of naked cuticle and denticle diversity (Nusslein-Volhard and Wieschaus 1984). Genetic analysis of a number of *Drosophila* mutants possessing phenotypes that are similar to or opposite to *wg* mutants led researchers to order a number of genes that are downstream targets of Wg signaling in a genetic pathway. Since then, the biochemical functions of the protein products of many of these genes, as well as an ever-increasing number of additional proteins that function in the Wg signal transduction pathway, have been elucidated (Dierick and Bejsovec 1999).

The functions of Wg signaling in a variety of other developing fly tissues has also been examined. For instance, during *Drosophila* development, dorsal expression of Wg functions to pattern the heart and dorsal muscles (Lawrence et al. 1995; Wu et al. 1995). *wg* mutants lack a subset of heart precursor cells and display muscle defects. For example, Eve protein expression in pericardial and dorsal muscle 1 cells is lost in *wg* mutant *Drosophila* embryos, indicating that Wg signaling is required for the proper specification of these cells

(Lawrence et al. 1995; Wu et al. 1995). Downstream components of the Wg signal transduction cascade, such as *dishevelled (dsh)* and *armadillo (arm)*, mediate the Wg signal required for mesodermal patterning (Park et al. 1996).

Recent studies have also indicated that Wg is required for proper patterning of the *Drosophila* retina. In the fly eye, during the process of ommatidial morphogenesis, rows of photoreceptor clusters differentiate in a succession that begins at the posterior margin and continues anteriorly. The morphological marker for this progression is the morphogenetic furrow, an indentation which is formed by the contraction of cells as they undergo the process of differentiation; the furrow moves across the eye disc in a posterior to anterior direction (Wolff and Ready 1993). Early Wg expression in the dorsal and ventral margins of the eye disc functions to regulate the development of these cells into adult head tissue and to inhibit the morphogenetic furrow from initiating improperly at the lateral margins of the disc. Loss of *wg* has been associated with loss of adult head structures, as well as ectopic furrow initiation in the lateral margins of the eye. Wg also appears to inhibit furrow progression. If Wg is expressed ectopically in the center of the disc, morphogenetic furrow progression is blocked (Chanut and Heberlein 1997; Ma and Moses 1995; Treisman and Rubin 1995). Furthermore, Wg plays a role in the dorsal/ventral patterning of the eye disc. When Wg expression in the eye disc is manipulated, the dorsal/ventral axis of the disc shifts, as indicated by changes in the expression domains of asymmetric markers, the position of the site of morphogenetic furrow initiation, the pattern of epithelial growth, and the chirality of ommatidial clusters (Heberlein et al. 1998; Ma and Moses 1995; Wehrli and Tomlinson 1998). Thus, Wg signaling functions in numerous aspects of *Drosophila* retinal development.

Studies in *Drosophila* prompted a number of researchers to examine the function of orthologues of *wg*, other *Wnt* genes, and genes that function up- and downstream of *wg* in many different organisms. These studies have led to a better understanding of Wnt functions in a variety of developmental processes in both vertebrates and invertebrates. For example, Wnt-7a functions in vertebrate limb patterning. Loss of *Wnt-7a* results in the ventralization of mouse limbs (Parr and McMahon 1995). Furthermore, the *Caenorhabditis elegans Wnt* gene *mom-2* and a number of its downstream targets have been isolated. In the nematode, Wnt signaling appears to regulate blastomere polarity (Rocheleau et al. 1997; Thorpe et al. 1997). Although the functions of a number of *Wnt* genes and genes that function downstream of Wnt signaling during the development of a variety of creatures are being elucidated, comparatively little is known about the functions of Wnt signaling during development of organisms more closely related to *Drosophila*, such as crustaceans. Since Wnt proteins function to pattern a variety of developing tissues, analyzing *Wnt* gene expression could lead to a better understanding of pattern formation in crustaceans and allow for examination of the patterning of crustacean tissues at the molecular level. Collecting these types of data may also lead to a better understanding of arthropod and *Wnt* gene evolution.

A *wg (Wnt-1)* orthologue was cloned from the malacostracan crustacean *Mysidium columbiae*. Hereafter, although we refer to *Mysdium columbiae* as "mysids," our comments refer to this mysid species, and not necessarily to all species of mysids. The developmental expression pattern of the *M. columbiae wingless (mcowg)* gene was examined through in situ hybridization. During mysid development, expression of *mcowg* is detected in dorsal tissues and in the developing eye. Despite the fact that *mcowg* expression is detected in only a small subset of the tissues that express the *wg* gene during *Drosophila* development, the dorsal and eye expression patterns of *Drosophila wg* and *mcowg* are quite similar. These observations provide molecular evidence that the processes of heart, muscle, and retinal patterning are conserved between insects and crustaceans.

Materials and methods

Animals

M. columbiae were collected from their natural habitat in Belize. For RNA isolation, adult animals with brood pouches were placed directly in 95% ethanol following collection. For expression analysis, adults, including females with embryos, were fixed for 15–30 min in 4% formaldehyde in PEM, as described previously (Patel et al. 1989). Following fixation, the animals were stored in absolute methanol for subsequent in situ or immunohistochemical analysis.

Cloning

A degenerate PCR approach was used to isolate a clone corresponding to the 3′ end of the *mcowg* coding region. RNA was isolated with Trizol (GibcoBRL) from a collection of embryos spanning a variety of developmental stages. cDNA was synthesized from 4 µg of mysid RNA with the Superscript Preamplification System (GibcoBRL). Degenerate primers corresponding to the protein sequences ECKCHGM (forward primer 5′-A,A/G T,G,T/C A,A,A/G T,G,T/C C,A,T/C G,G,T/C/A/G A,T,G-3′) and FNWCCHV (reverse primer 5′-T/C/A/G,T,C/G A/G,C,A A/G,C,A C,C,A A/G,T,G A/G,C,A-3′) were used to amplify mysid cDNA. PCR was completed according to the guidelines suggested in the Superscript Preamplification System (Gibco BRL). Each 50-µl reaction contained one-tenth of the cDNA synthesis reaction, 1.5 mM $MgCl_2$, 20 mM TRIS-HCl, 50 mM KCl, 10 mM dNTPs, 0.2 µM primers, and 5 U *Taq* polymerase (Boehringer Mannheim). Following thermocycling in a Perkin-Elmer thermocycler (5 min denaturation at 95°C preceding 40 cycles of 30 s denaturation at 95°C, 1.5 min annealing at 48°C, and 1.5 min extension at 72°C), PCR products were gel purified, cloned, and sequenced. A BLAST search indicated that a 408-bp clone (not including primers) encoded a novel *wg* gene, which was named *mcowg*.

Gene-specific primers corresponding to the most 5′ region of the 408-bp clone were synthesized for use with the 5′RACE System for Rapid Amplification of cDNA Ends (GibcoBRL). This system allowed for the isolation of additional, more 5′ DNA fragments, the longest of which was approximately 1.2 kb in size, from mysid cDNA. These fragments were cloned and sequenced; BLAST searches indicated that a number of the clones (all of which overlapped at the 3′ end) consisted of *wg- (Wnt-1-)* related DNA corresponding to the 5′ end of the *mcowg* gene. The largest

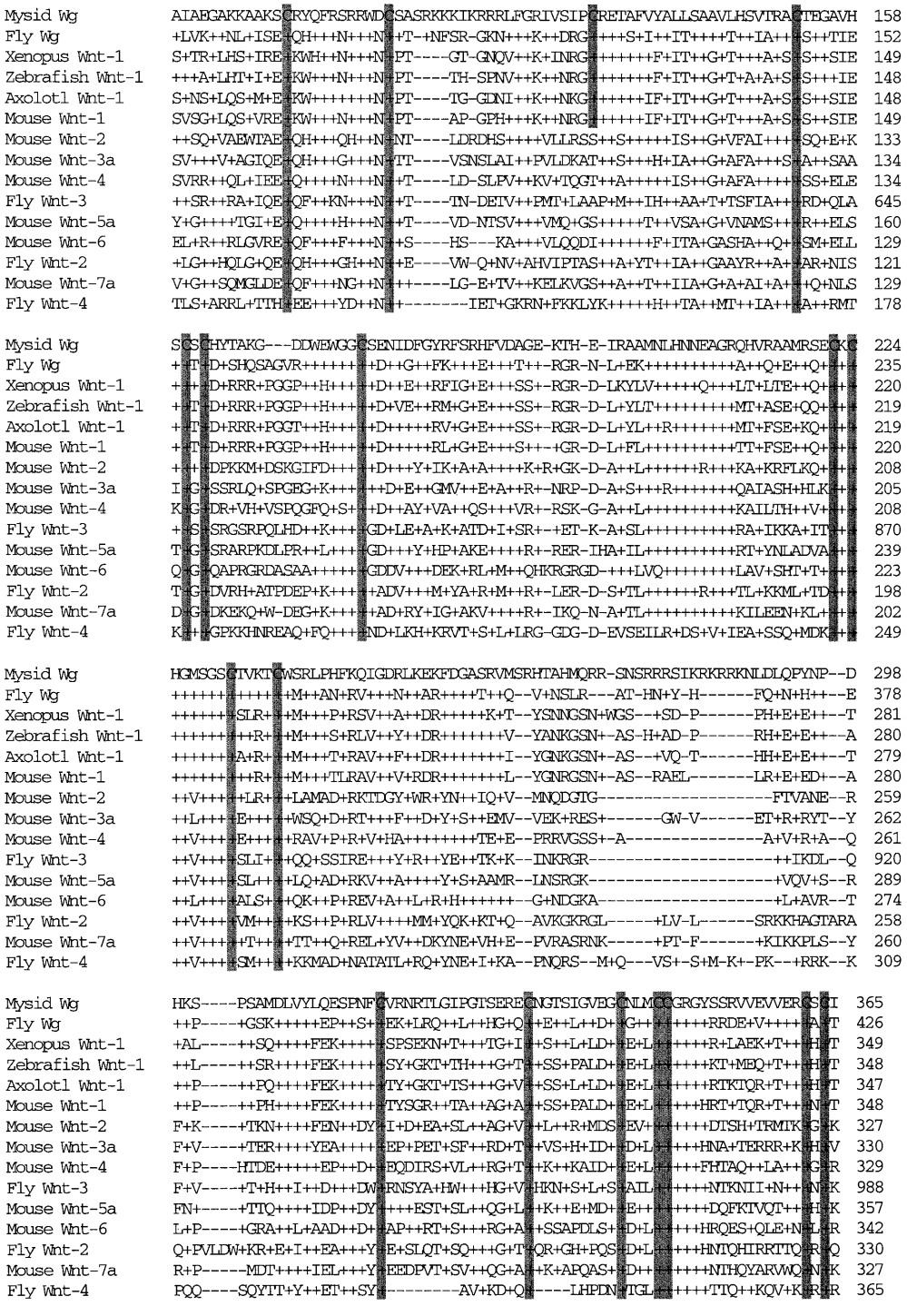

Fig. 1A, B *Mcowg* encodes a Wg (Wnt-1) orthologue. **A** Alignment of Mcowg to other Wnt proteins is shown. Mcowg shows a high degree of sequence identity to all of the Wnt proteins shown, but it most closely resembles Wnt-1 (Wg) proteins. *Plus signs* (+) denote identity and *hyphens* (-) indicate gaps in alignment. Conserved cysteine residues, including the Wnt-1-specific mysid C130 residue, are *highlighted*. The alignment shown in this figure was generated by adding the Mcowg protein sequence to alignments that had been published previously by Sidow (1992; alignment can be viewed at http://www.stanford.edu/~rnusse/sidow.html) and Graba et al. (1995). **B** An unrooted parsimony tree including Mcowg, fly, and mouse Wnt proteins is shown. Parsimony analysis indicates that Mcowg is a Wg orthologue. In this analysis, other Wnt proteins grouped with their respective orthologues (mouse Wnt-7a and fly Wnt-2; mouse Wnt-5a and fly Wnt-3; Sidow 1992). Bootstrap values for each clade are shown as percentages

RACE clone incorporated the rest of the 5′ *mcowg* coding region as well as the 5′ UTR. In all, the degenerate PCR and RACE clones of *mcowg* spanned the 5′ UTR and nearly the entire coding region of the *mcowg* gene.

Due to the fact that ventral neuroectodermal expression of the *mcowg* gene was not detected, it was hypothesized that a gene duplication event (resulting in the production of two *wg* genes in mysids whose combined function would perform functions similar to those performed by a single *Drosophila wg* gene) may have occurred. In an attempt to find a second *mcowg* gene, three additional degenerate PCR reactions (same conditions as above) were performed. Multiple clones (at least five) from the original and each of the three subsequent PCR reactions were sequenced. The sequences of all of the clones corresponded to either the original *mcowg* gene or to a novel mysid *Wnt* gene which, through protein sequence alignment and phylogenetic tree analysis, was designated a *Drosophila Wnt-3* orthologue (Duman-Scheel and Patel, unpublished data). Each separate PCR reaction produced both of these two gene products. In all, of 45 clones sequenced, 19 corresponded to the original *mcowg* gene and 18 corresponded to the *M. columbiae Wnt-3 (Mcownt-3)* orthologue. Sequences of both *mcowg* and *mcownt-3* were deposited in GenBank (accession nos. AF438206 and AF438207, respectively). Thus, although the primer sets could amplify a *Drosophila Wnt-3* orthologue from mysids,

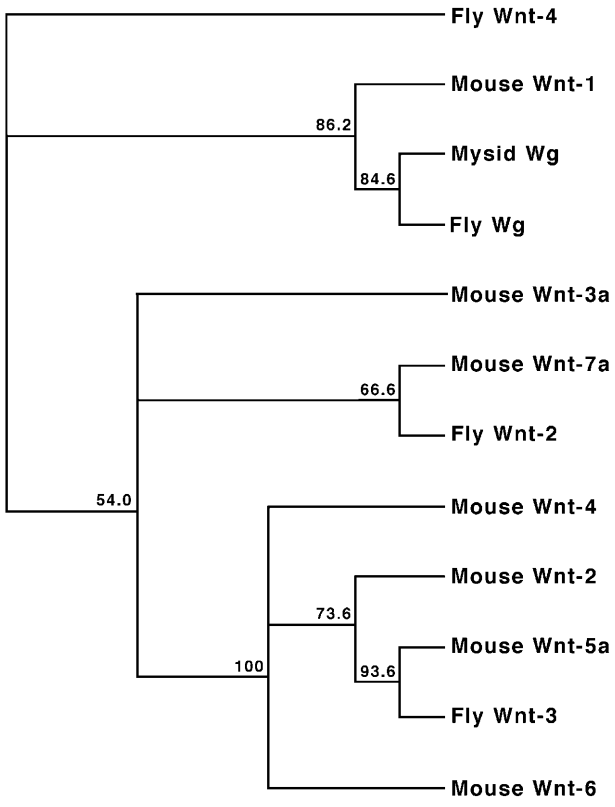

Fig. 1B (Legend see page 116)

they did not amplify a second mysid *wg* gene. Additional attempts to use different degenerate PCR primers or to use the original degenerate primers at a reduced stringency failed to produce products of the size expected for members of the *Wnt* gene family.

In order to be certain that the degenerate PCR and RACE clones described above also corresponded to a single *mcowg* gene, a number of gene-specific primers deduced from the sequence of the RACE and degenerate PCR clones were used to amplify various sized products from mysid cDNA. In all cases, single products of the various expected sizes were produced. Some of these products were cloned, sequenced, and found to span the original degenerate PCR and 5′ RACE clones. The ability to amplify these DNA fragments indicated that the degenerate PCR and 5′ RACE clones did in fact correspond to a single *mcowg* gene.

Sequence analysis

The Wnt protein sequence alignment completed by A. Sidow was used (Sidow 1992; alignment can be viewed at Wnt protein web site: http://www.stanford.edu/~rnusse/sidow.html). D Wnt-4 protein was aligned to these sequences based on the published alignment of this protein to other *Drosophila* Wnt proteins (Graba et al. 1995). Mcowg protein was aligned to Wnt protein sequences using Clustal V implemented in the MacVector program package. Final adjustments were made by eye. Non-conserved N-termini and insertions were not included in the alignment. In addition to the N-termini, amino acid residues 207–214 in mouse Wnt-5a, 140–160 in mouse Wnt-6, 164–173 and 281–364 in fly Wg, 134–139 in fly Wnt-2, 0–450 and 689–842 in fly Wnt-3 were not included. References for published Wnt protein sequences include: Busse et al. 1990; Gavin et al. 1990; Graba et al. 1995; van den Heuvel et al. 1993; McMahon and McMahon 1989; Molven et al. 1991; Noordermeer et al. 1989; Roelink and Nusse 1991; Russell et al. 1992; Van Ooyen and Nusse 1984.

SEQBOOT was used to produce >1,000 bootstrapped data sets from the alignments of Mcowg, fly, and mouse Wnt proteins. The phylogeny estimate for each bootstrapped data set was generated using PROTPARS (Protein Sequence Parsimony Method). These data were used as the input for CONSENSE, which generated the unrooted majority-rule consensus parsimony tree with bootstrap values shown in Fig. 1B. Branches of the tree were collapsed if bootstrap values were not significant (<50%). SEQBOOT, PROTPARS, and CONSENSE are part of the Phylip 3.5c software package (by J. Felsenstein). A variety of other software programs produced similar results (not shown).

In situ hybridization

Mysids that had been stored in methanol were rehydrated and embryos were dissected from brood pouches. Younger embryos were dissected away from their yolk and older embryos were sonicated for 1 s in a Branson 250 Sonifier set at its lowest power setting. A digoxygenin-labeled riboprobe corresponding to a 0.9-kb fragment that included most of the *mcowg* coding region was synthesized according to the Boehringer Mannheim protocol. In situ hybridization was performed using a modified version (Davis et al. 2001) of the protocol described by Patel (1996). Most significantly, the proteinase K step was replaced by treatment of embryos for 30 min with a detergent solution containing 1% sodium dodecyl sulfate (SDS); 0.3% SDS was added to the hybridization and wash solutions.

Immunohistochemistry

In some cases, following in situ hybridization with the *mcowg* probe, animals were rinsed briefly in phosphate-buffered saline with 0.1% Triton X-100 (PT), blocked in PT plus 5% normal goat serum, and stained with the anti-Engrailed (En) monoclonal antibody (mAb) 4D9 (Patel et al. 1989) according to the Patel (1994a) protocol. This protocol was also used to stain embryos with the anti-Eve 2B8 antibody (Patel et al. 1994). For Eve staining, older embryos (just prior to dorsal closure) were sonicated for 2 s with a Branson 250 Sonifier set at its lowest power setting.

For visualization of Wg expression in *Drosophila* eye discs, imaginal discs were dissected from the *wg-lac Z* line (corresponds to wg^{en11} in Ma and Moses 1995), fixed, and stained with anti-ß-galactosidase (ß-gal) antibody (Cappell) and 22C10 mAb (Fujita et al. 1982; stains neural cells) according to the Patel (1994a) protocol.

Relative staging of mysid and fly embryos

Mysid embryos were staged relative to fly embryos using two criteria: (1) the appearance of Engrailed (En) stripes in the neuroectoderm and (2) the formation of various Eve- and En-expressing homologous neurons (Duman-Scheel and Patel 1999).

Results

Cloning of the *mcowg* gene

Briefly, a degenerate PCR approach was used to clone a DNA fragment from mysid embryonic cDNA, and 5′ rapid amplification of cDNA ends was used to obtain larger fragments of the gene. BLAST searches indicated that these clones most closely resembled Wnt-1 (*wg*) DNA, and the gene was subsequently named *mcowg*. In all, just over 1.6 kb corresponding to nearly the entire open reading frame and all or part of the 5′ UTR of the *mcowg* gene

were obtained. A start methionine following several in-frame stop codons was identified. The first 25 amino acids following the start methionine correspond to a signal sequence with the predicted charged N-, hydrophobic H-, and polar C-regions (von Heijne 1985). In all, the cDNA clones obtained included 1,095 bp of coding sequence corresponding to 365 amino acids. Based on its alignment to other Wnt proteins, the Mcowg protein probably contains an additional 22 amino acids at its C-terminus encoded by DNA which has not yet been cloned.

Figure 1A shows the alignment of Mcowg protein to other Wnt proteins. The non-conserved amino termini are not shown, and non-alignable insertions have been removed. Although a high degree of amino acid identity is seen among all of the Wnt proteins listed in Fig. 1A, the Mcowg protein resembles other Wg (or Wnt-1) proteins more closely than the other Wnt proteins listed. In the regions aligned in Fig. 1A, Mcowg has the highest percentage identity to *Drosophila* Wg (57%). It has a higher percentage identity to other Wnt-1 proteins (50–53% identity to Wnt-1 proteins listed in Fig. 1) than it has to other *Drosophila* Wnt proteins (40% identity to *Drosophila* Wnt-2, 44% identity to *Drosophila* Wnt-3, and 35% identity to *Drosophila* Wnt-4). Other mouse Wnts are 42–48% identical to the Mcowg protein. Parsimony analysis indicates that *Mcowg* is a *wg* orthologue (Fig. 1B).

All Wnt family proteins contain 22 conserved cysteine residues (Nusse and Varmus 1992; Sidow 1992). Four of these cysteines are at the C-terminus in a region corresponding to part of the *mcowg* gene that was not cloned, but all of the other 18 cysteine residues (highlighted in Fig. 1A) are conserved in the Mcowg protein. The Mcowg protein also contains an additional cysteine, C130, which is unique to Wnt-1 proteins. Furthermore, the spacing between cysteines in Wnt proteins is largely invariant (Nusse and Varmus 1992; Sidow 1992), and this conserved spacing between cysteine residues is observed in the Mcowg protein.

Dorsal expression of *mcowg*

mcowg expression was examined through in situ hybridization. *mcowg* transcripts were first detected in early germband stage embryos at the most-lateral regions of each hemisegment (Fig. 2A). At this stage (prior to dorsal closure), these lateral-most regions (which are lateral to where limbs will form) correspond to the dorsal-most tissue of the embryo. Interestingly, although 12 En protein stripes can be detected in the mysid ventral neuroectoderm at this time (Patel 1994b; Fig. 2B), no ventral neuroectodermal expression of *mcowg* is observed. This is unexpected because striped expression of Wg and En are dependent upon each other in *Drosophila* embryos (Cadigan and Nusse 1997). Also, ventral neuroectodermal stripes of *wg* expression have been detected in a number of other arthropods, including the beetle *Tribolium castaneum* (Nagy and Carroll 1994), the cricket *Gryllus bimaculatus* (Niwa et al. 2000), the grasshopper *Schistocerca gregaria* (Dearden and Akam 2001), and the branchiopod crustacean *Triops longicauditus* (Nulsen and Nagy 1999). Additionally, expression of *mcowg* at the anterior and posterior termini is not detected at this stage.

Dorsal cells expressing *mcowg,* which appear to be located in the mesoderm, are still detected as the process of dorsal closure begins in the embryo. For a brief period, a continuous domain of *mcowg* expression that extends across the boundaries of segments is visible. This continuous domain of expression persists in anterior-most segments, although in most segments *mcowg* expression is later restricted to dorsal patches of cells within each hemisegment (Fig. 2C). Dorsal *mcowg* expression fades prior to hatching (not shown).

The dorsal *mcowg* expression pattern was compared with the dorsal expression pattern of *Drosophila wg*. During *Drosophila* development, dorsal expression of Wg functions to pattern the mesoderm. *wg* mutants lack a subset of heart precursor cells and have muscle defects (Lawrence et al. 1995; Wu et al. 1995). Wu et al. (1995) determined that Wg functions to ensure proper heart precursor cell formation shortly after gastrulation, during the period of approximately 4–4.5 h after egg laying. *wg* expression in a *Drosophila* embryo of this age is shown in Fig. 2D. The embryo in Fig. 2D has been flattened such that the lateral-most regions correspond to the dorsal portion of the embryo. The earliest stage of *mcowg* expression in the most-lateral region of the embryo (Fig. 2A, B) likely corresponds to the period when Wg is required for heart formation in *Drosophila*. In later stages (stage 12 and later), *Drosophila* Wg expression is also restricted to the dorsal portion of the segment, but the role of Wg in heart formation occurs prior to this time.

In *Drosophila*, a subset of pericardial cells as well as dorsal muscle 1 cells are marked by expression of the Eve protein (Fig. 2E; Azpiazu and Frasch 1993; Bodmer 1993; Frasch et al. 1987; Park et al. 1996). These cells are found dorsally in each hemisegment. Upon dorsal closure, Eve-expressing pericardial cells, as well as other pericardial cells and the inner myosin-expressing contractile cardiac cells migrate dorsally, merge at the dorsal midline, and give rise to the dorsal heart vessel (Bodmer et al. 1990). Eve expression in pericardial and dorsal muscle 1 cells is lost in *wg* mutant *Drosophila* embryos, suggesting that Wg signaling is required for their proper specification (Lawrence et al. 1995; Wu et al. 1995). A similar set of dorsal Eve-expressing cells (Fig. 2F) is detected at a corresponding time period during mysid development, suggesting that mysids possess homologous mesodermal derivatives. In mysids, heart precursor cells (marked by arrows in Fig. 2F) are found only in thoracic segments, while Eve-positive dorsal muscle cells (arrowheads in Fig. 2F) are found in both thoracic and abdominal segments. Interestingly, dorsal Eve-expressing cells have been identified in all insects examined to date (N. Patel, unpublished data). Detection of conserved dorsal *wg* and Eve expression provides molecular evidence indicating that the processes of heart and muscle cell spec-

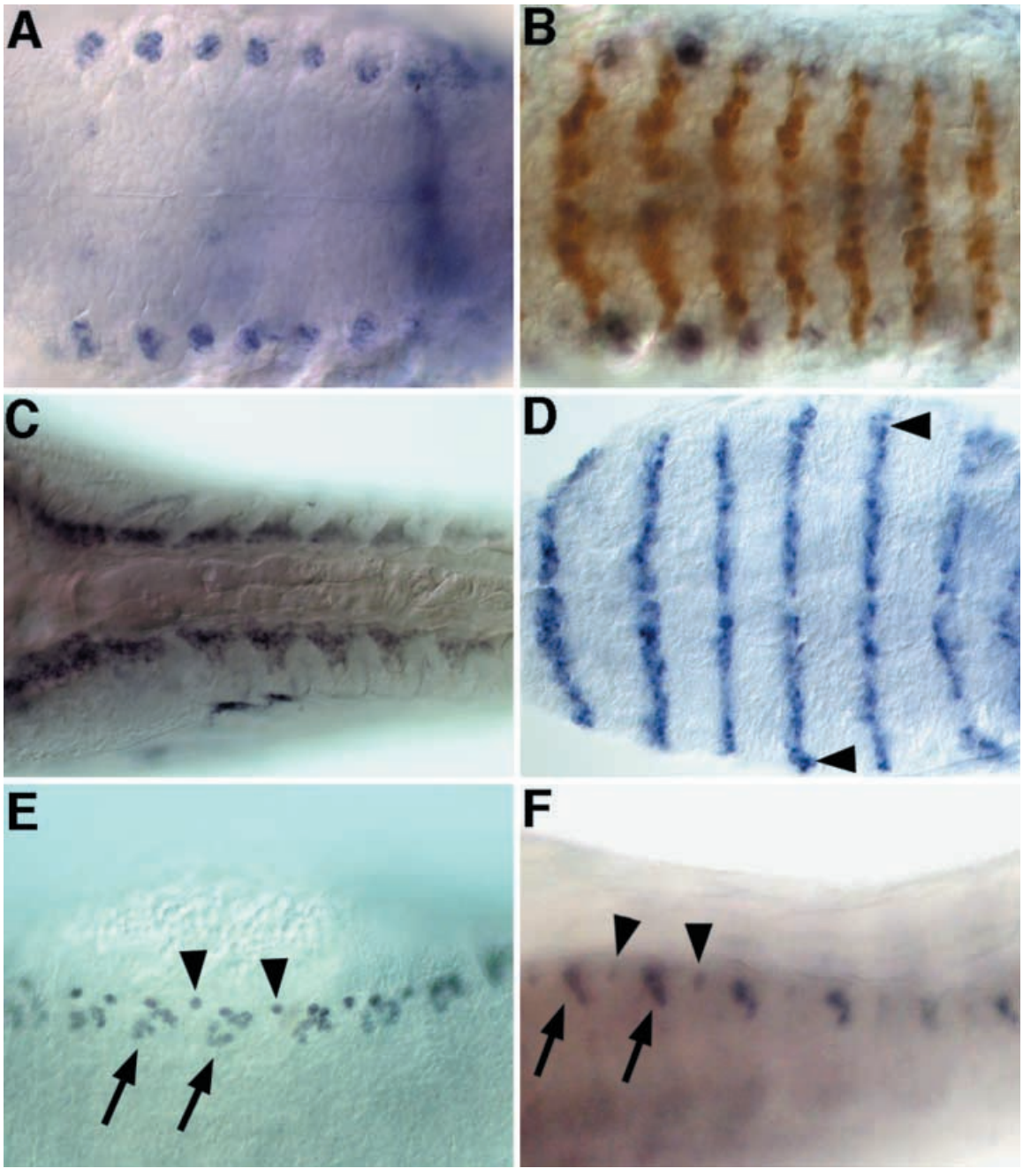

Fig. 2A–F Conserved heart and muscle patterning functions of Wg. Expression of *mcowg* was analyzed through in situ hybridization. Anterior is to the left in all figures. Ventral views are shown in **A** and **B**. *mcowg* transcripts are first detected in the lateral-most regions (corresponding to the dorsal portions) of each segment in mysid embryos (**A**). Surprisingly, although dorsal *mcowg* transcripts (*blue*) and ventral neuroectodermal En (*brown*) protein stripes (twelve in this embryo) can be detected at this stage (**B**), neuroectodermal stripes of *mcowg* expression are not observed. A dorsal view of an older mysid embryo is shown in **C**. During the process of dorsal closure, on either side of the yolk, continuous domains of *mcowg* expression extending across segmental boundaries are detected (still visible in only the anterior-most segments in **C**), but *mcowg* expression is eventually restricted to dorsal patches of cells within each hemisegment (as seen in the posterior segments in **C**). In *Drosophila* embryos, approximately 4–4.5 h after egg laying (**D**), *wg* (*blue*) functions to pattern the mesoderm (Wu et al. 1995). In **D**, a ventral view of a fly embryo is shown, and *arrowheads* mark *wg* expression at the lateral (dorsal) portion of hemisegments; this dorsal *wg* expression is comparable to the *mcowg* expression shown in **A** and **B**. Later, the ventral and dorsal expression domains of *Drosophila wg* split, and the dorsal expression domain of *wg* more closely resembles that observed in mysids. In *Drosophila* (lateral view of a stage 13 embryo is shown in **E**), Eve expression in a subset of pericardial cells (*arrowheads*) as well as dorsal muscle 1 cells (*arrows*; Azpiazu and Frasch 1993; Bodmer 1993; Frasch et al. 1987; Park et al. 1996) is dependent on earlier expression of *wg* (**D**). An identical set of dorsal Eve-expressing cells can be detected in mysids (lateral view shown in **F**). These data indicate that the processes of heart and muscle cell specification are likely to be conserved in distantly related arthropods

ification are likely to be conserved in distantly related arthropods.

Expression of *mcowg* in the eye

mcowg is expressed during development of the mysid eye. *mcowg* eye expression is initially detected in four clusters of cells, two clusters in each hemisphere of the mysid head (Fig. 3A). Eventually, the two clusters of cells in each hemisphere are connected by an arch of *mcowg*-expressing cells (Fig. 3B–D). Although the initial clusters of *mcowg*-expressing cells are located on the outer surface of the head (Fig. 3A), the arch of *mcowg*-positive cells connecting the two clusters is initially located on the medial (inner) surface of the head. When

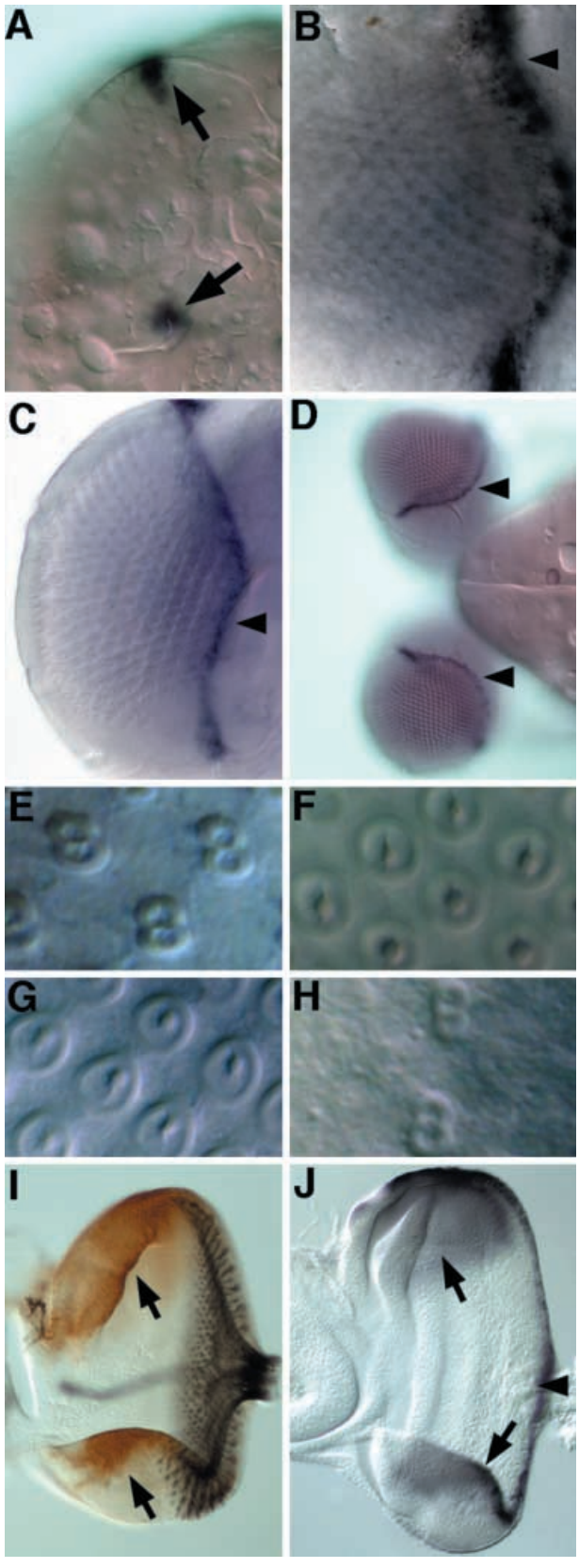

Fig. 3A–J Conserved retinal patterning functions of Wg. In situ hybridization was used to detect expression of *mcowg* during mysid eye development. In **A–D**, mysids of progressively older ages have been oriented so that *mcowg* expression can be compared with expression of Wg in *Drosophila* eye discs (**I,J** discs oriented anterior to the left and dorsal up). *mcowg* eye expression is initially detected in two clusters of cells (marked by *arrows*) located on the surface of each hemisphere of the mysid head (**A** anterior portion of the head is up). Ommatidial formation initiates on the inner surface of the head (not visible in the plane of focus shown in **A**). Later, the two clusters of cells in each hemisphere are connected by an arch of *mcowg*-expressing cells (*arrowheads* in **B–D**) located in the plane where ommatidia are forming (left of *mcowg* expression in **B–D**). *mcowg*-expressing arch cells bound the ommatidia on one side. Initially, *mcowg*-expressing arch cells and ommatidia are found on the medial surface of the mysid head and are more easily viewed when the optic lobe is dissected away from the yolk and laid down flat (**B** and **C**). During retinal morphogenesis, the optic lobe gradually everts. Eventually, the once medial photoreceptor region rotates to a more external position, and the *mcowg* arch cells are located at the posterior portion of the eye (**D** anterior is to the left). The expression pattern of *mcowg* in the developing mysid eye resembles ß-galactosidase expression in the third instar *Drosophila* eye disc of *wg-lac Z* reporter lines (**I, J**). In flies (**I**), Wg expression (*brown*, marked by *arrows*) in the dorsal and ventral margins of the eye disc (Baker 1988) functions to restrict furrow initiation to the posterior margin of the disc (photoreceptor cells in **I** are labeled by the anti-neural 22C10 antibody, *black*). This expression pattern is comparable to the early *mcowg* expression pattern shown in **A**, although the domains of Wg expression are broader in the fly. Similar to mysids (**B–D**), as retinal morphogenesis progresses in flies, an arch of Wg expression (*black*, marked by *arrowhead* in **J**) extending across the posterior margin of the eye disc connects the original two domains of expression (*arrows*) in the dorsal and ventral margins of the eye disc and bounds the ommatidia at the posterior portion of the eye. In flies, retinal morphogenesis progresses in a posterior to anterior direction. At the end of morphogenesis, the most recently formed row of ommatidia is located at the anterior portion of the disc, and the oldest row of ommatidia is bounded by Wg expression in the posterior margin. This also appears to be true in mysids. Newly formed mysid ommatidia are shown in **E**, which is a high magnification photo of the young embryo pictured in **B**. Once mature, ommatidia take on a different appearance (**F** a high magnification shot of mature embryo pictured in **D**), which likely reflects the differentiation of crystalline cone. In the embryo shown in **C** (which is older than **B** and younger than **D**), ommatidia located near the *mcowg*-expressing arch cells have a mature appearance (high-magnification shot shown in **G**). However, newly formed ommatidia (**H**) can be visualized in the region farthest from the domain of *mcowg* expression. These newly formed ommatidia (**H**), which will eventually be located at the anterior portion of the mysid eye, have formed more recently than ommatidia closer to the domain of *mcowg* expression (which will eventually be located in the posterior portion of the mysid eye, **G**)

the optic lobe is dissected away from the yolk and laid down flat so that the once medial cells can be viewed more easily (Fig. 3B, C), rows of ommatidia that are found in the same plane as the arch of *mcowg*-expressing cells are visible. The arch of *mcowg* expression persists as retinal morphogenesis proceeds (progressively older embryos are shown in Fig. 3B–D). By the end of retinal morphogenesis, the once medial photoreceptor region everts to an external position (Fig. 3D), and the arch of *mcowg*-expressing cells bounds the ommatidia at the posterior portion of the eye. At this time, a second arch of *mcowg* expression (barely visible in Fig. 3D) can be visualized below the surface of the photoreceptor cells within the brain.

The expression pattern of *mcowg* in the developing eye resembles Wg expression in the *Drosophila* eye disc (Fig. 3I, J). In third instar *Drosophila* larvae, as morphogenetic furrow initiation and progression begins, Wg expression is found in the dorsal and ventral margins of the eye disc (Baker 1988; Fig. 3I). Later, when the morphogenetic furrow has progressed anteriorly and many ommatidial clusters are visible, an arch of Wg expression extending across the posterior margin of the eye disc connects the original two domains of expression in the dorsal and ventral margins (Fig. 3J). It seems likely that the two initial dorsal and ventral marginal domains of expression in the fly (Fig. 3I) are equivalent to the two initial domains of expression found on the surface of each mysid head hemisphere (Fig. 3A). However, the domains of *wg* expression are not as broad in mysids as they are in the fly eye disc. The arch of *wg* expression in the posterior margin of the fly eye disc that connects the dorsal and ventral marginal domains of *wg* expression (arrowhead in Fig. 3J) corresponds to the arch of *mcowg* expression (Fig. 3B–D) connecting the original two domains of expression in the mysid eye (Fig. 3A).

Paulus (1979) suggested that the eyes of mandibulates are homologous (Osorio and Bacon 1994). Furthermore, Friedrich and Benzer (2000) determined that the role of wg during eye development is conserved in insects. Due to the similarity between *wg* expression patterns in the *Drosophila* and mysid eye, we hypothesized that the role of *wg* during eye development is conserved between insects and crustaceans. In the fly eye, ommatidial morphogenesis (marked by the morphogenetic furrow) initiates at the posterior margin (Fig. 3I) and progresses anteriorly (Wolff and Ready 1993). Wg expression in the dorsal and ventral margins (Fig. 3I) restricts furrow initiation to the posterior margin and prevents progression of the morphogenetic furrow into inappropriate regions of the eye disc (Chanut and Heberlein 1997; Ma and Moses 1995; Treisman and Rubin 1995).

Several lines of evidence indicate that the processes of retinal morphogenesis and the retinal patterning functions of Wg may be similar in flies and mysids. During retinal morphogenesis, ommatidial clusters can be visualized on the apical surface of the mysid eye. Although there is no visible morphogenetic furrow in mysids, new rows of photoreceptor cells appear to be added sequentially, just as they are in the fly eye disc. For example, in eyes of the progressively older mysid embryos shown in Fig. 3B–D, 8, 18, and 21 rows of ommatidia can be visualized, respectively. When retinal differentiation initiates, the initial two domains of *mcowg* expression are detected (Fig. 3A; differentiating photoreceptor clusters that are located on the medial surface of the head are not visible in this plane of focus). This expression pattern is consistent with the idea that at this stage of eye development, Mcowg functions to limit the domains of the eye field, just as it does in *Drosophila* (Chanut and Heberlein 1997; Ma and Moses 1995; Treisman and Rubin 1995).

As retinal morphogenesis progresses in *Drosophila*, the most recently formed rows of ommatidia are located in the anterior portion of the eye. The rows of ommatidia that were formed first are located in the posterior portion of the eye and are eventually bounded by a posterior domain of *wg*-expressing cells (the function of late posterior Wg expression is unknown). A similar phenomenon occurs in *M. columbiae*. The rows of ommatidia that were formed first are eventually bounded by *mcowg*-expressing arch cells in the posterior, and rows of ommatidia are added anterior to these rows. This point is illustrated in Fig. 3E–H. A high-magnification view of newly formed ommatidia from the young embryo in Fig. 3B is shown in Fig. 3E. More-mature ommatidia from the older embryo in Fig. 3D have a different appearance (high-magnification view shown in Fig. 3F) that most likely reflects the progression of crystalline cone differentiation. The embryo pictured in Fig. 3C (which is of an intermediate age) has both newly formed ommatidia, as well as more-mature ommatidia. In this embryo, ommatidia near the *mcowg*-expressing arch cells (which will eventually be located at the posterior portion of the eye) have a mature appearance (Fig. 3G). More recently formed ommatidia are found in the region farthest from the *mcowg*-expressing arch cells (Fig. 3H) in what will become the anterior portion of the eye. Sagittal sectioning of mysid eyes following *mcowg* in situ hybridization also indicates that ommatidial clusters closest to the arch of *mcowg* expression appear to be more mature than ommatidial clusters located farther away from the arch of *mcowg* expression (data not shown). Conservation of the spatial relationship between *mcowg* expression and the progression of retinal development indicates that the functions of Wg in retinal patterning are likely to be conserved between insects and crustaceans.

Discussion

Cloning of the *mcowg* gene from *M. columbiae* and characterization of its expression pattern are presented here. This analysis provides molecular evidence that the processes of heart, muscle, and retinal patterning are conserved among distantly related arthropods. Dorsal *wg* expression, which functions to pattern heart and muscle cells in *Drosophila* (Lawrence et al. 1995; Wu et al. 1995), is conserved in mysids (Fig. 2A, B, D). Like

Drosophila Wg, McoWg likely induces expression of Eve in pericardial and dorsal muscle 1 cells (Fig. 2E, F). Furthermore, conservation of the eye expression pattern of *wg* in mysids and flies (Fig. 3) suggests that the functions of Wg in retinal patterning are conserved between insects and crustaceans. Like *Drosophila* Wg, Mcowg may inhibit improper initiation of neural differentiation (thus promoting formation of head tissue) and function to pattern the dorso-ventral axis of the mysid retina. Thus, although *mcowg* is expressed in only a subset of the tissues in which *Drosophila wg* is expressed, its function is likely to be conserved within these tissues.

Developing ways to disrupt gene expression or to mis-express genes during crustacean development will be useful. It would be interesting to see if manipulating *mcowg* expression affects heart and muscle formation or retinal patterning in crustaceans. Until then, cloning additional genes that function to pattern the *Drosophila* mesoderm or eye and analyzing their expression patterns in relation to that of *mcowg* could be informative.

mcowg expression was surprisingly not detected in a number of mysid tissues. Most notably, since the *wg* orthologue in the branchiopod crustacean *Triops longicaudatus* is expressed in the ventral neuroectoderm and the limbs (Nulsen and Nagy 1999), one would have expected to detect *mcowg* expression in these tissues. On the other hand, the *Triops* gene is not expressed dorsally. Expression of *wg* has not yet been analyzed during formation of the eye in older *Triops*, so it is not known if *wg* expression patterns similar to those reported here would be observed.

Based on the differences in the *Triops* and mysid *wg* expression patterns, it is possible that a gene duplication event might have resulted in the production of two *wg* genes in crustaceans. The tasks performed by a single *wg* gene in *Drosophila* could be split between two genes in crustaceans. However, a second *mcowg* gene was not found in *M. columbiae*. It remains possible that a second *mcowg* gene does exist, but these experiments do not support this explanation.

Alternatively, perhaps *wg* function is not required for patterning the mysid ectoderm or limbs. This would be surprising, particularly in the light of the recent *Triops* results (Nulsen and Nagy 1999). However, one could imagine ways to dispense with a requirement for Wg in these tissues. For example, in *Drosophila*, aside from the period when En expression is dependent on Wg expression, there are periods when En expression is regulated by pair-rule genes (Martinez Arias 1993) and when En autoregulates its own expression (Heemskerk et al. 1991). Perhaps mysid En expression is regulated directly by pair-rule orthologues, and then by autoregulation.

Another explanation for these results is that a different member of the Wnt family may have replaced *wg* function in particular tissues. The function of a number of additional Wnt family genes, including *D Wnt-2*, *D Wnt-3* (also known as *D Wnt-5*), and *D Wnt-4*, has been studied in *Drosophila* (Graba et al. 1995; Kozopas et al. 1998; Russell et al. 1992). Mysid orthologues of these other *Drosophila Wnt* genes might be expressed in the ectoderm or limbs and could potentially substitute for the lack of *wg* gene expression in these mysid tissues. A mysid *D Wnt-4* orthologue could be the most likely candidate, as *D Wnt-4* maps close to *Drosophila wg*, and the same *cis*-regulatory elements are thought to promote overlapping expression patterns of both genes during embryogenesis (Gieseler et al. 1995; Graba et al. 1995). Although these genes seem to have taken on antagonistic (Gieseler et al. 1999) or distinct (Buratovich et al. 2000) functions during the development of some fly tissues, *DWnt 4* can rescue loss-of-function *wg* phenotypes in the antennal and haltere discs and can substitute for *wg* during specification of the wing field (Gieseler et al. 2001). Thus, a mysid orthologue of *D-Wnt 4* might function to pattern the ectoderm or limbs. Cloning and analyzing the expression patterns of additional mysid *Wnt* genes will lead to a better understanding of the evolution of *Wnt* gene function in arthropods.

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