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Expression of a *Wnt* Gene in Embryonic Epithelium of the Leech

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A new member of the *Wnt* class of cell-cell communication molecules was identified in the leech *Helobdella triserialis*, on the basis of a conserved 86 amino acid coding sequence and exon structure. This gene, *htr-wnt-A*, is not an obvious homolog of any one of the previously described *wnt* class proteins.

The embryonic expression of *htr-wnt-A* has been characterized at the cellular level, using nonradioactive *in situ* hybridization and polyclonal antibodies generated via a novel method of antigen presentation. Subcellular localization of the *htr-wnt-A* protein was examined by the use of immunofluorescence and confocal microscopy. *htr-wnt-A* is among the first zygotically expressed genes in *Helobdella*, appearing first in a single cell of the eight-cell embryo. In early development it is expressed within a stereotyped subset of micromeres and later, in a seemingly dynamic and stochastic pattern, by cells in a micromere-derived provisional embryonic epithelium. Its spatial and temporal expression pattern make it a candidate for participation in the regulation of cell fate in the O/P equivalence group.

INTRODUCTION

During embryonic development, communication between cells provides critical signals that orchestrate processes such as pattern formation, morphogenesis, and differentiation. Some embryonic communication pathways utilize specific proteins as intercellular signaling molecules (Mercola and Stiles, 1988). One group of proteins thought to serve as developmental signaling proteins are those belonging to the *Wnt* family. *Wnt* proteins share sequence homology as well as conserved structural features, such as an N-terminal signal sequence in the absence of hydrophobic transmembrane domains, and a large number of conserved cysteine residues (van Ooyen and Nusse, 1984; van Ooyen *et al.*, 1985; Wainwright *et al.*, 1988; McMahon and McMahon, 1989; Gavin *et al.*, 1990). *Wnt*-1, the most extensively studied of the *Wnt* family proteins, enters the secretory pathway (Brown *et al.*, 1987; Papkoff *et al.*, 1987; McMahon and Moon, 1989) where it is processed and glycosylated (Brown *et al.*, 1987; Papkoff *et al.*, 1987; Papkoff, 1989; Bradley and Brown, 1990; Papkoff and Schryver, 1990) then secreted (Papkoff, 1989; Bradley and Brown, 1990; Papkoff and Schryver, 1990). Although the *Wnt*-1 protein lacks the hydrophobic region characteristic of integral membrane proteins, it does not behave as a freely diffusible protein in cell culture experiments. The protein is instead tightly associated with the cell surface and/or the extracellular matrix (Bradley and Brown, 1990; Papkoff and Schryver, 1990). While a receptor and signaling pathway for a *Wnt* protein has yet to be identified it is known that *Wnt* protein expression can either directly or indirectly modulate gap junction-mediated cell-cell communication (Olson *et al.*, 1991).

The evolutionary origins of the prototype *Wnt* gene predates the Cambrian radiation, as evidenced by the presence of the *Wnt* family members in vertebrates (van Ooyen and Nusse, 1984; van Ooyen *et al.*, 1985; Noordermeer *et al.*, 1989; Molven *et al.*, 1991), arthropods (Rijsewijk *et al.*, 1987), and nematodes (Kamb *et al.*, 1989). Molecular and genetic studies of *Wnt*-1 in mouse (McMahon and Bradley, 1990; Thomas and Capecchi, 1990) and its homolog *wingless* in *Drosophila* (Nusslein-Volhard and Wieschaus, 1980; Baker, 1987; Rijsewijk *et al.*, 1987) have shown that *Wnt*-1 and *wingless* are essential for normal embryogenesis and that these and other *Wnt* family genes are expressed during early development (Shackleford and Varmus, 1987; Wilkinson *et al.*, 1987; McMahon and McMahon, 1989; Gavin *et al.*, 1990; Christian *et al.*, 1991; Molven *et al.*, 1991). Some murine *Wnt* genes exhibit distinct and complex patterns of transcript accumulation (Shackleford and Varmus, 1987; Wilkinson *et al.*, 1987; McMahon and McMahon, 1989; Gavin *et al.*, 1990).

Thus, characterization of *Wnt* proteins and their expression patterns in embryos of other organisms could both serve to identify sites of critical embryonic cell-cell interactions and also shed light on the evolution of the *Wnt* gene family. We have chosen to examine the embryonic expression pattern of *Wnt* proteins in glossiphoniid leeches, annelids whose development has been studied in great detail using classic embryological techniques. We report here the isolation and embryonic expression pattern of one leech *Wnt* gene, *htr-wnt-A*. During early development the expression of this gene is restricted to a subset of micromeres. Anti-*htr-wnt-A* antibodies reveal a subcellular distribution consistent with a protein that enters the secretory pathway yet remains closely

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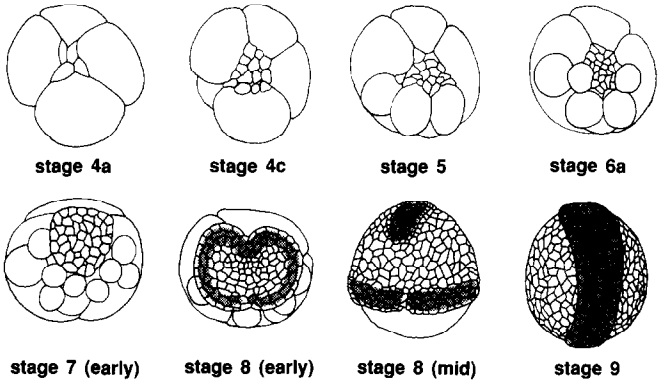

FIG. 1. Drawings showing selected stages of embryonic development in glossiphoniid leeches. Except for the stage 9 embryo (lower right), all drawings view the animal pole, including the prospective dorsal surface. (Top row, left to right) Stage 4a (eight-cell embryos): macromere D′ is toward the bottom, micromere d′ is at lower left of the quartet of small cells. Stage 4c; DNOPQ‴, the ectodermal precursor, is toward the bottom and the dnopq″, dnopq‴, and dnopq′ micromeres lie just above it, from left to right, respectively. Stage 5; left M teloblast is at left. Right M teloblast lies beneath the left and right NOPQ cells, toward the bottom. Stage 6a; left and right OPQ cells are toward the bottom, left and right N teloblasts are just above them on either side of the micromere cap. (Bottom row, left to right) Early stage 7 embryo: all teloblasts have formed and begun to produce blast cells, which lie under the epithelium derived from the micromere cap. Early stage 8; the heart-shaped germinal bands (stippling) have begun to coalesce along the future ventral midline, still covered by the squamous micromere-derived epithelium. Mid stage 8: coalescence movements of the germinal bands (stippling) into the germinal plate are accompanied by epiboly of the sheet of micromere-derived cells comprising the provisional epithelium. Epithelial cells lying over the germ bands and germinal plate have smaller surface areas. Anterior end of the germinal plate is visible at the top of the embryo; left and right germinal bands lie at its equator, beneath the leading edge of the epithelium. Ventral view of early stage 9: the germinal plate is complete and segmental tissues are forming, including the segmental ganglia of the ventral nerve cord (heavy outline). Subsequently, definitive epidermis arises from the germinal plate and expands laterally at the expense of the provisional epithelium.

associated with the expressing cell. Later during embryonic development, *htr-wnt-A* is expressed by cells in a transient micromere-derived embryonic epithelium that has been shown to be essential for the proper differentiation of a neighboring cell lineage (Ho and Weisblat, 1987). We have, in addition, observed that *htr-wnt-A* is expressed ectopically in spontaneously occurring developmentally abnormal embryos.

MATERIALS AND METHODS

Embryos

Helobdella triserialis and *Helobdella robusta* embryos were obtained from laboratory colonies maintained in 1% artificial sea water on a diet of physid snails (Weisblat *et al.*, 1980). *Placobdella sp.* embryos were obtained from a leech collected from the wild. Embryos were staged as previously described (Fernandez, 1980; Stent and Weisblat, 1982; Weisblat and Blair, 1984).

Occasional embryos deviate from the normal pattern of early stereotyped cleavages and such embryos almost always fail to complete development (D.A.W. and others, unpublished observations). Moreover, occasional clutches of embryos show an abnormally high incidence of such aberrant early cleavages, and in these "aberrant clutches," even apparently normal embryos often fail later in development. In the experiments reported here all the embryos from aberrant clutches were considered separately from embryos originating from clutches exhibiting normal development and viability.

DNA Amplification

One degenerate deoxyoligonucleotide, 5′ GA(T/C)TGG(G/C)A(G/A/T/C)TGGGG(G/A/T/C)GG(G/A/T/C)TG 3′ (oligo A), was chosen to represent the sense strand coding for the conserved amino acid sequence DW(E/H)WGGC, which is found at residues 164-170 in *Wnt*-1 (van Ooyen and Nusse, 1984; van Ooyen *et al.*, 1985) and residues 179-185 in *wingless* (Rijsewijk *et al.*, 1987). A second degenerate deoxyoligonucleotide, 5′ GC(T/C)TC(G/A)TT(G/A)TT(G/A)TG(G/A/T/C)A-G(G/A)TTCAT 3′ (oligo B), was chosen to represent the antisense strand corresponding to the conserved amino acid sequence MNLHNNEA, residues 198-205 in *Wnt*-1 and residues 213-220 in *wingless*. Amplifications of genomic DNA were carried out essentially as described by Kamb *et al.* (1989). A 20-μl solution containing *Helobdella triserialis* chromosomal DNA (1 μg), oligo A (1 μg) and oligo B (1 μg), 10 m*M* Tris-HCl (pH 8.3), 50 m*M* KCl, 2 m*M* $MgCl_2$, 0.01% gelatin, 100 μ*M* dNTPs, and 1 unit of Taq DNA polymerase was incubated in a thermocycler (Perkin-Elmer) for 40 cycles: 1 min at 94°C, 1 min at 50°C, and 2 min at 55°C.

The oligonucleotide 5′ TAGATCTCAGCAAGGAGT-CAGCGTT 3′ (oligo C) was designed to introduce a *Bgl*II site at the amino terminus of the *htr-wnt-A* coding exon when used in PCR in conjunction with oligo B. Amplifications using oligo B and oligo C were performed as for oligo A and oligo B except that 100 pg of a genomic λ clone containing *htr-wnt-A* was substituted for the chromosomal DNA and 20 cycles of amplification were used instead of 40.

DNA Cloning and Analysis

Upon completion, polymerase chain reaction (PCR) amplification mixtures were added to a 20-μl solution of 50 m*M* Tris-HCl (pH 7.5), 10 m*M* $MgCl_2$, 1 m*M* DTT, 100 μ*M* dNTPs, 1 m*M* ATP, 5 units of T4 DNA kinase (NEB), and 2 units of T4 DNA polymerase (NEB) and incubated

A

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                  10        20        30                                  40        50        60                  70        80
                   :         :         :                                   :         :         :                   :         :
htr-wnt-A  SKESAFVSAARSAGIRYAITRACSRSDLAGCACDKS                          ISKKDWEWNGCSANVNYGIKFCENFLDAREKY          SDNSQSLMNLHNNRAGRK
           * ***** *  ***  ********  **  * ****                           ** **** ****** *****   *  ****            ** **************
Wg    (38) CRETSFIYAITSAAVTHSIARACSEGTIESCTCDYSHQSRSPQANHQ               AGSVAGVRDWEWGGCSDNIGFGFKFSREFVDTGER          GRNLREKMNLHNNEAGRA
Wnt-1 (36) CRETAFIFAITSAGVTHSVARSCSEGSIESCTCDYRR                        RGPGGPDWHWGGCSDNIDFGRLFGREFVDSGEK          GRDLRFLMNLHNNEAGRT
Wnt-2 (49) SRESAFVYAISSAGVVFAITRACSQGELKSCSCDPKK                        KGSAKDSKGTFDWGGCSDNIDYGIKFARAFVDAKER        KGKDARALMNLHNNRAGRK
Wnt-3 (36) TRESAFVHAIASAGVAFAVTRSCAEGTSTICGCDSHH                        KGPPGEGWKWGGCSEDADFGVLVSREFADAREN           RPDARSAMNKHNNEAGRT
Wnt-4 (43) TREAAFVYAISSAGVAFAVTRACSSGELEKCGCDR                          TVHGVSPQGFQWSGCSDNIAYGVAFSQSFVDVRERSKGAS    SSRALMNLHNNEAGRK
Wnt-5a(38) SRETAFTYAVSAAGVVNAMSRACREGELSTCGCSR                          ARPKDLPRDWLWGGCGDNIDYGHPFAKEFVDARERERIHAKGSYESARILMNLHNNEAGRR
Wnt-5b(39) SRETAFTYAVSAAGVVNAISRACREGELSTCGCSR                          AARPKDLPRDWLWGGCGDNVEYGYRFAKEFVDAREREKNFAKGSEEQGRALMNLQNNEAGRR
Wnt-6 (34) IRETAFVFAITAAGASHAVTQACSMGELLQCGCQAPRGRAPPRPSGLLGTPGPPGPTGSPDASAAWEWGGCGDDVDFGDEKSRLFMDAQHK      RGRGDIRALVQLHNNEAGRL
Wnt-7a(43) SREAAFTYAIIAAGVAHAITAACTQGNLSDCGCDKEKQGQY                    HWDEGWKWGGCSADIRYGIGFAKVFVDAREI             KQNARTLMNLHNNEAGRK
Wnt-7b(44) SREAAFTYAITAAGVAHAVTAACSQGNLSNCGCDREKQGYY                    NQAEGWKWGGCSADVRYGIDFSRRFVDAREI             KKNARRLMNLHNNEAGRK
Xwnt-8(37) TRETSFVHAISSAGVMYTLTRNCSMGDFDNCGCDDSR                        NGRIGGRGWVWGGCSDNAEFGERISKLFVDGLET          GQDARALMNLHNNEAGRL

                                                                        DWEWGGC                                     MNLHNNEA
                                                                          H
                                                                        oligo A                                     oligo B
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B

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          10        20        30        40        50        60        70        80        90        100       110       120
           :         :         :         :         :         :         :         :         :         :         :         :
TTGGGTGAAAATAAATTCATTTTTTTATGAACCTGTGAACTTGCAGCCAGCAAGGAGTCAGCGTTCGTTTCAGCAGCGAGGTCAGCTGGCATTCGATACGCCATCACGAGAGCCTGCAGT
                                                S  K  E  S  A  F  V  S  A  A  R  S  A  G  I  R  Y  A  I  T  R  A  C  S

AGGAGCGACCTGGCCGGATGTGCGTGCGACAAGTCCATCTCCAAAAAAGACTGGGAGTGGAACGGATGCAGTGCGAACGTCAACTACGGAATTAAGTTCTGCGAAAACTTTTTAGACGCC
R  S  D  L  A  G  C  A  C  D  K  S  I  S  K  K  D  W  E  W  N  G  C  S  A  N  V  N  Y  G  I  K  F  C  E  N  F  L  D  A

CGTGAGAAATATTCAGACAACTCACAGTCGCTGATGAACCTGCACAACAATCGAGCTGGTCGCAAGGTTGGGCTCTCAAATGTTTCTTGCTTGATTGAGTTTATTTTAAAAATAATGATT
R  E  K  Y  S  D  N  S  Q  S  L  M  N  L  H  N  N  R  A  G  R  K
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FIG. 2. Comparison of *Wnt* family genes and sequence from *htr-wnt-A*. (A) The putative amino acid sequence of a characterized portion of *htr-wnt-A* is shown aligned with the corresponding portions of other *Wnt* proteins, the *htr-wnt-A* residues are numbered and discontinuities in the sequences were introduced to maintain sequence alignment and maximize homology with *htr-wnt-A*. Residue identities between *htr-wnt-A* and one or more *Wnt* protein are shown in bold face, the number of identities for a given *Wnt* protein is shown in parentheses and their positions are marked by an asterisk. Below the *Wnt* alignments are shown the sequences to which the corresponding degenerate oligonucleotides, oligo A and oligo B, were designed to amplify the intervening coding region of *htr-wnt-A*. The source of the *Wnt* sequences are *Wnt*-1 (van Ooyen and Nusse, 1984); *Wnt*-2 (McMahon and McMahon, 1989); *Wnt*-3 (Roelink *et al.*, 1990); *Wnt*-4 -5a -5b -6 -7a and -7b (Gavin *et al.*, 1990); *Xwnt*-8 (Christian *et al.*, 1991). (B) The sequence of a portion of *Helobdella triserialis* genomic DNA encoding a phylogenetically conserved exon of *htr-wnt-A*. The amino acid sequence encoded is shown below and sequences similar to the consensus sites (Mount, 1982) for metazoan splice acceptors (C,U)AG(G,A) and splice donors (C,A)AGGU(A,G)AGU are shown in bold face.

at 37°C for 1 hr. The incubation was terminated by phenol extraction and ethanol precipitation and the mixture used directly in ligation reactions with *Sma*I cut pBluescript (Stratagene) or into pUC119 (Vieira and Messing, 1987) which had been cut with *Hin*cII and *Hin*dIII and treated with Klenow fragment in the presence of dNTPs. All other DNA cloning and manipulations were performed essentially as described in Maniatis *et al.* (1982).

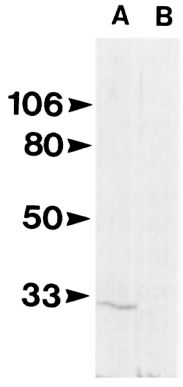


FIG. 3. Immunoblot analysis of whole embryo protein extracts from stages 7 and 8 *H. triserialis* embryos. (A) Immunoblot performed with purified anti-*htr-wnt-A* antibody. (B) Control immunoblot in which anti-*htr-wnt-A* antibodies were omitted. The positions of the molecular weight standards and their molecular weights in kilodaltons are indicated at left.

DNA sequences were determined by the dideoxynucleotide method, modified for use with T7 DNA polymerase (Tabor and Richardson, 1987) on double-stranded plasmid DNA (Sequenase, United States Biochemicals).

Isolation of htr-wnt-A Genomic Clones

EMBL3 λ phage (Frischauff *et al.*, 1983) carrying *htr-wnt-A* genomic sequence were isolated from a *H. triserialis* genomic DNA library (Wedeen *et al.*, 1990) using a ^{32}P-labeled RNA probe generated from a pBluescript clone of the oligo A-oligo B PCR product. Positive phage were restriction mapped and a representative phage isolate was subcloned as an *Eco*RI fragment into pBluescript to obtain a 4-kb plasmid clone of genomic DNA encompassing the PCR-amplified sequence.

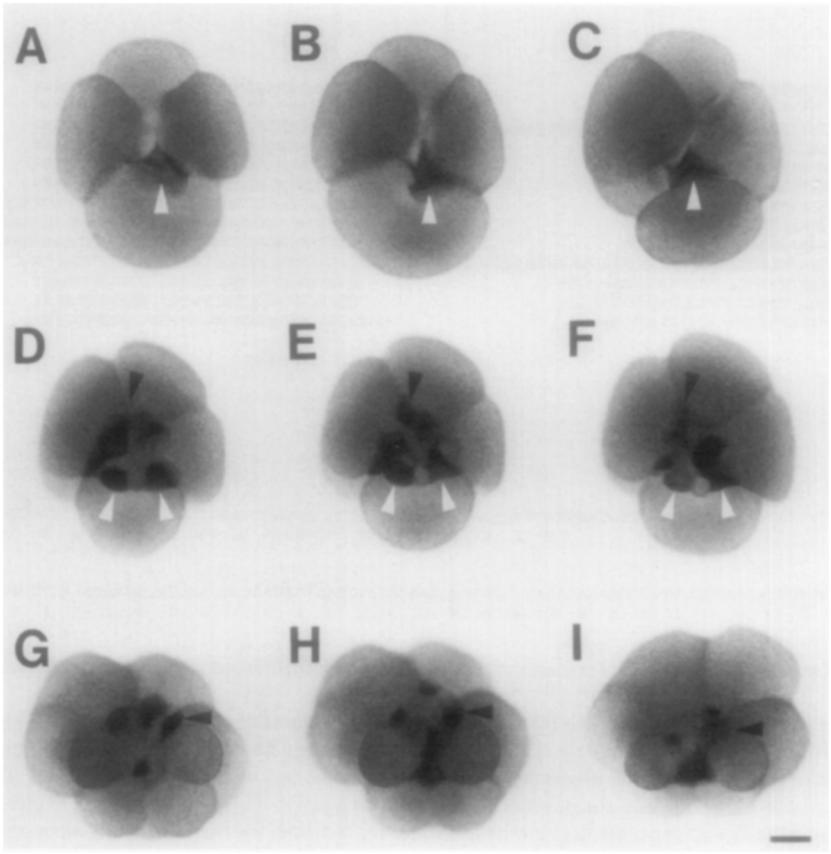


FIG. 4. Immunolocalization of *htr-wnt-A* antigen in stages 4a, 4c and 6a *Helobdella triserialis* embryos, using HRP-conjugated second antibody and DAB visualization. (A–C) Stage 4a embryos in which the D′ macromere (bottom) is in the process of dividing to generate DNOPQ and DM. Micromere d′ (white arrowheads) is stained in each of these embryos. (D–F) Stage 5 embryos in which cell DNOPQ‴ (bottom) is beginning to cleave. Micromeres dnopq′ and dnopq″ are stained in each embryo (white arrowheads), while the staining of other, unidentified micromeres (black arrowheads) is variable. (G–I) Stage 6a embryos (the left and right OPQ proteloblasts toward the bottom), showing marked embryo to embryo variability in *htr-wnt-A* expression among micromeres (e.g., black arrowheads). Scale bar, 100 μm.

Fusion Protein Expression and Purification

A pUC119 clone that contained the 83 amino acid long portion of *htr-wnt-A* protein coding region, amplified using oligo B and oligo C, was iterated in tandem as follows. One aliquot of this plasmid was cut with *Bgl*II and *Eco*RI and a second aliquot cut with *Bam*HI and *Eco*RI. The two digests were mixed, ligated, and transformed into *recA-E. coli* (XL-1, Stratagene). Approximately one of four transformants were the result of ligation of the *Bgl*II site to the *Bam*HI site resulting in a tandem in-frame duplication of the *htr-wnt-A* coding sequence. This duplication process was repeated several times yielding plasmids containing 4, 8, and 16 in-frame repeats.

A plasmid (pZ/16xhtr-wnt-A) was constructed that fused *lacZ* to the 16-times-repeated *htr-wnt-A* coding region by transferring the *htr-wnt-A* sequences into pUR298 (Ruther and Muller-Hill, 1983). The resultant fusion protein, β-D-galactosidase fused to 16 *htr-wnt-A* repeats (βgal/16xhtr-wnt-A), was produced at high levels by *Escherichia coli* (XL-1) harboring pZ/16xhtr-wnt-A upon induction with 0.5 m*M* IPTG. The βgal/16xhtr-wnt-A released from cells by sonication was insoluble in solutions containing less than 8 *M* urea. Sonicated cell lysates were centrifuged and the insoluble pellet was dispersed in 4 *M* urea using a Dounce homogenizer and centrifuged to remove solubilized materials. The pellet, containing the majority of the βgal/16xhtr-wnt-A, was resuspended in 8 *M* urea and centrifuged. The supernatant was dialyzed against PBS and the precipitate that formed was greater than 90% βgal/16xhtr-wnt-A as assayed by SDS-PAGE. The yield was approximately 100 mg/liter of culture.

A plasmid (pE/4xhtr-wnt-A) which fused the *E. coli trpE* gene to four *htr-wnt-A* repeats was constructed by transferring the four-times-repeated *htr-wnt-A* reading frame into pATH10 (Koerner *et al.*, 1990). The resultant fusion between anthranilate synthase and four *htr-wnt-A* repeats (AS/4xhtr-wnt-A) was induced as described

(Koerner *et al.*, 1990) by addition of indolacrylic acid (Sigma) to *E. coli* (XL-1) harboring pE/4xhtr-wnt-A grown in minimal media (Maniatis *et al.*, 1982). AS/4xhtr-wnt-A was purified to greater than 90% homogeneity by the same procedure used to purify βgal/16xhtr-wnt-A. The yield of AS/4xhtr-wnt-A per liter of culture was approximately 25 mg.

Antibody Production and Purification

Insoluble βgal/16xhtr-wnt-A was used as an antigen for the production of a polyclonal antiserum in a rabbit (Babco) essentially as described by Harlow and Lane (1988). The rabbit was immunized on Day 1 with 500 μg protein in complete Freund's adjuvant, then boosted 3, 6, and 9 weeks after the initial injection with 250 μg protein in Freund's. Serum, collected 10.5 weeks after the first immunization, reacted strongly to AS/4xhtr-wnt-A, not to unfused anthrylate synthase and only marginally to β-D-galactosidase and *E. coli* proteins as assayed by immunoblots of purified proteins and *E. coli* lysates (data not shown).

The anti-βgal/16xhtr-wnt-A serum was immunoaffinity purified (Harlow and Lane, 1988). An affinity column was made by coupling 10 mg of purified AS/4xhtr-wnt-A to 1 ml of Affigel-10 (Bio-Rad) in the presence of 8 *M* urea. Antibodies specific for *htr-wnt-A* were bound to the column, the column was extensively washed, and *htr-wnt-A* antibodies were eluted with 100 m*M* glycine (pH 2.5). The eluted antibodies were subjected to a second round of binding and elution. The resultant anti-*htr-wnt-A* antibodies were specific for the *htr-wnt-A* coding region and exhibited no affinity to endogenous *E. coli* proteins, as assessed by immunoblotting, or to the *wingless* gene product, as assayed by its inability to immunostain *Drosophila* embryos expressing the *wingless* gene product (van den Heuvel *et al.*, 1989) (data not shown).

Immunoblot Analysis of Embryonic Proteins

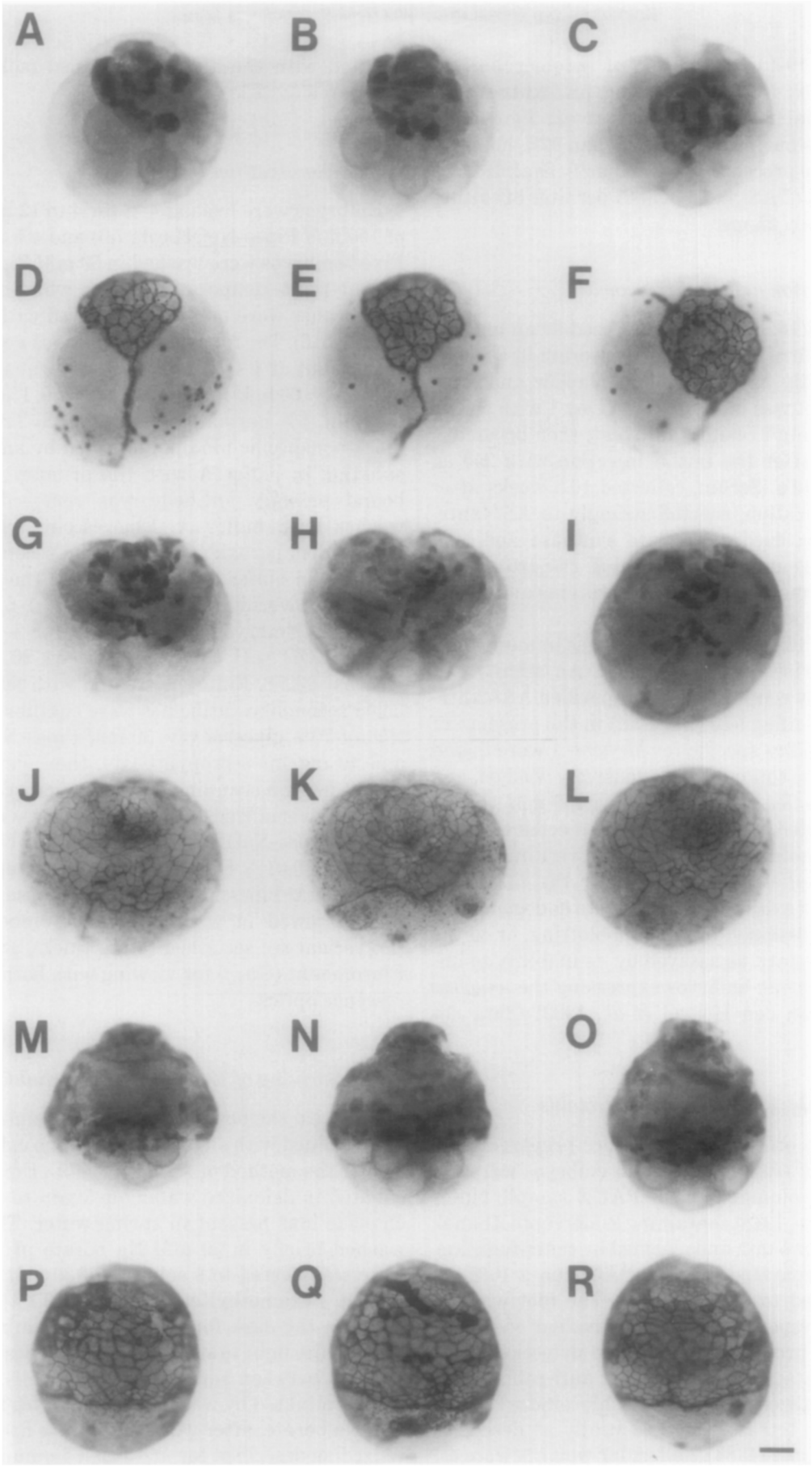
Whole embryo protein samples were prepared from stages 7 and 8 *H. triserialis*. Settled embryos were homogenized in 5 volumes of SDS-PAGE sample buffer (Harlow and Lane, 1988) containing 2-mercaptoethanol, heated to 95°C for 5 min and clarified by centrifugation. Proteins were separated by SDS-PAGE on a 10% gel and electroblotted to nitrocellulose. The blot was cut into strips (corresponding to the equivalent of 40 embryos), blocked, processed for antigen detection using alkaline phosphatase-conjugated goat anti-rabbit antibody, and visualized with bromochloroindoyl phosphate/nitro blue tetrazolium, essentially as described by Harlow and Lane (1988). Molecular weights were estimated with the aid of prestained molecular weight standards (BRL).

Immunolocalization in Embryos

Embryos were fixed at 4°C for 4 to 12 hr in a solution of 100 m*M* Pipes-NaOH (pH 7.0) and 4% formaldehyde. Fixed embryos were washed in 50 m*M* Pipes-NaOH (pH 7.0) at room temperature, after which their vitelline membranes were manually removed with forceps (Dumont No. 5). The following incubations and washes were carried out at 4°C. Embryos were incubated for 4 hr in buffer A (100 m*M* Pipes-NaOH pH 7.0, 1% bovine serum albumin, 1% normal goat serum, 1% Triton X-100) to block nonspecific binding, followed by an overnight incubation in buffer A with the primary antibody. Unbound primary antibody was removed by six 1-hr washes with buffer A. Fluorescein-, rhodamine-, or horseradish peroxidase-conjugated secondary antibody (Cappel) in buffer A was added and the embryos were incubated overnight, followed by six 1-hr washes with buffer A. Embryos were washed for 1 hr in 50 m*M* Pipes-NaOH (pH 7.0), 0.1% Tween 20, and 1 μg/ml Hoechst 33258. Embryos treated with fluorescently labeled secondary antibodies were equilibrated in a solution of 70% glycerol v/v, 50 m*M* Pipes-NaOH (pH 7.0), and 40 mg/ml *n*-propylgallate, then viewed in whole-mount using a scanning confocal microscope (Bio-Rad). HRP was visualized by reacting embryos in a solution of 50 m*M* Pipes-NaOH (pH 7.0), 400 μg/ml diaminobenzidine, and 0.01% H_2O_2, followed by extensive washing with 50 m*M* Pipes-NaOH (pH 7.0). These embryos were either viewed in whole-mount or embedded in JB-4 (Polysciences), sectioned (4-μm thick), and mounted in Fluromount (Gurr) for viewing with both DIC and fluorescence optics.

Silver Staining of Embryonic Epithelial Cells

To reveal the boundaries of superficial cells, embryos were stained with silver methenamine using a modification of the method of Arnolds (1979). Embryos were incubated in deionized water for 5 min to remove excess chloride ions present in spring water. They were then washed briefly in 30 m*M* Na borate pH 7.5. Embryos were transferred to a solution of 30 m*M* Na borate pH 7.5, 1% hexamethyltetramine, and 0.1% $AgNO_3$, incubated in the dark for 5 min then illuminated with intense white light to stimulate silver deposition along the furrows between surface epithelial cells. The reaction was terminated by washing the embryos for 5 min in 30 m*M* Na borate, after which they were fixed and devitellinized as described for *htr-wnt-A* immunolocalization.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In Situ Analysis

Digoxygenin-labeled strand-specific *in situ* probes were prepared as follows. A 20-μl solution containing 100 ng of plasmid DNA (containing an insert to be labeled and linearized at a site bordering one end of the insert), 100 ng of oligonuceotide primer (chosen to prime DNA synthesis through the insert starting from one end and terminating at the point of linearization), 10 m*M* Tris-HCl (pH 8.3), 50 m*M* KCl, 2 m*M* $MgCl_2$, 0.01% gelatin, 100 μ*M* dATP, 100 μ*M* dCTP, 100 μ*M* dGTP, 65 μ*M* dTTP, 35 μ*M* digoxygenin-11-dUTP (Boehringer-Mannheim), and 1 unit of Taq DNA polymerase was incubated in a thermocycler (Perkin-Elmer) for 80 cycles: 30 sec at 94°C, 30 sec at the oligonucleotide annealing temperature, and 1 min at 70°C. This labeling method permitted the synthesis of strand-specific DNA probes corresponding to either strand of Bluescript plasmids inserts, by using oligonucleotide primers corresponding to either the T7 or T3 promoter sequences (Stratagene, annealing temperature 40°C) that flank the multiple cloning sites and linearizing the plasmid template on either side of the insert.

In situ hybridization was carried out using procedures modified from those of Tautz and Pfeifle (1989). Embryos were fixed overnight at 4°C in a solution containing 4% paraformaldehyde and 0.1 *M* Pipes-NaOH, pH 7.2, then rinsed twice briefly in 50 m*M* Pipes-NaOH, pH 7.2, and placed at room temperature. Vitelline membranes were removed with forceps. Embryos were rinsed in PTW (50 m*M* Pipes-NaOH, pH 7.2, 0.1% Tween 20) and then incubated at room temperature for 10 min in PTW with 50 μg/ml proteinase K. Proteinase action was terminated by two 10-min washes in PTW with 2 mg/ml glycine. Embryos were washed in a 1:1 mixture of HYB:PTW (HYB: 50% formamide, 750 m*M* NaCl, 50 m*M* Pipes-NaOH, pH 7.2, 100 μg/ml single-stranded salmon sperm DNA, 50 μg/ml heparin, 1 m*M* EDTA, 0.1% Tween 20) for 30 min, then straight HYB for 30 min, and then prehybridized in HYB at 45°C for 1 hr. Digoxygenin-labeled probe (1 μg/ml) in HYB was incubated with the embryos for 18 hr at 45°C. Embryos were washed at 45°C in HYB, then HYB:PTW in the ratios 3:1, 1:1, and 1:3, followed by straight PTW for 30 min per solution. Subsequent steps were carried out at room temperature: incubation in PMTB (50 m*M* Pipes-NaOH, pH 7.2, 100 m*M* NaCl, 5 m*M* $MgCl_2$, 0.1% Tween 20, 1% BSA) for 1 hr; incubation in PMTB with alkaline phosphatase-conjugated anti-digoxygenin antibody (1:1000 dilution; Boehringer-Mannheim) for 2 hr; three 30-min washes with PNM. Embryos were washed two times for 10 min each in TMT (100 m*M* Tris-HCl, pH 9.5, 100 m*M* NaCl, 10 m*M* $MgCl_2$, 0.1% Tween 20). Alkaline phosphatase was visualized by a chromogenic reaction in TMT with X-phosphate (150 μg/ml) and tetrazolium blue (300 μg/ml). The color reaction was terminated by transferring the embryos to a solution of PTW containing 20 m*M* EDTA. Embryos were viewed in whole-mount.

RESULTS

Summary of Leech Development

Details of the early cleavages and general outlines of the later development of glossiphoniid leeches like *Helobdella* were first described by C. O. Whitman (1878, 1887, 1892) and have been extended in recent years (Weisblat *et al.*, 1984, Sandig and Dohle, 1988) (Fig. 1). Schemes for blastomere nomenclature and developmental staging have been devised and amended (e.g., Fernandez, 1980; Bissen and Weisblat, 1989). The 0.5-mm eggs of *H. triserialis* are fertilized internally, but do not begin cleaving until after they are deposited in cocoons on the ventral aspect of the parent (stage 0). Embryos can be removed from the cocoons at any time and raised to maturity in simple salt solutions. Cleavages are stereotyped and individual blastomeres can be identified by their size, their position in the embryo, the order in which they arise, and by the segregation of domains of yolk-deficient cytoplasm.

During cleavage (stages 1-6), three distinct classes of blastomeres are formed, *teloblasts*, *macromeres*, and *micromeres*. The teloblasts are five bilateral pairs of large stem cells designated M, N, O/P, O/P, and Q which ultimately give rise to all the segmentally iterated cells in the leech body. The macromeres, A‴, B‴ and C‴, are the three largest cells in the embryos by stage 6; they provide the substrate upon which the morphogenetic movements of embryogenesis take place and are eventually enveloped by the gut and digested during stages 9-11. The third class of cells, the micromeres, are small cells which form a cluster called the micromere cap at the animal pole (future anterior end) of the embryo and serve several roles in development. Most of the experimental results described here pertain to the micromeres and their progeny.

A total of 25 micromeres are produced by a highly stereotyped pattern of cell divisions during stages 4-6 of

FIG. 5. Localization of *htr-wnt-A* antigen and visualization of surface epithelium in stage 7 and 8 embryos of *Helobdella triserialis*. (A-C) Stage 7 embryos stained for *htr-wnt-A*. (D-F) Sibling embryos stained with silver to demarcate the cell boundaries of the epithelium. (G-I) Early stage 8 embryos stained for *htr-wnt-A* and siblings (J-L) stained with silver. (M-O) Mid stage 8 embryos stained for *htr-wnt-A* and siblings (P-R) stained with silver. Scale bar, 100 μm.

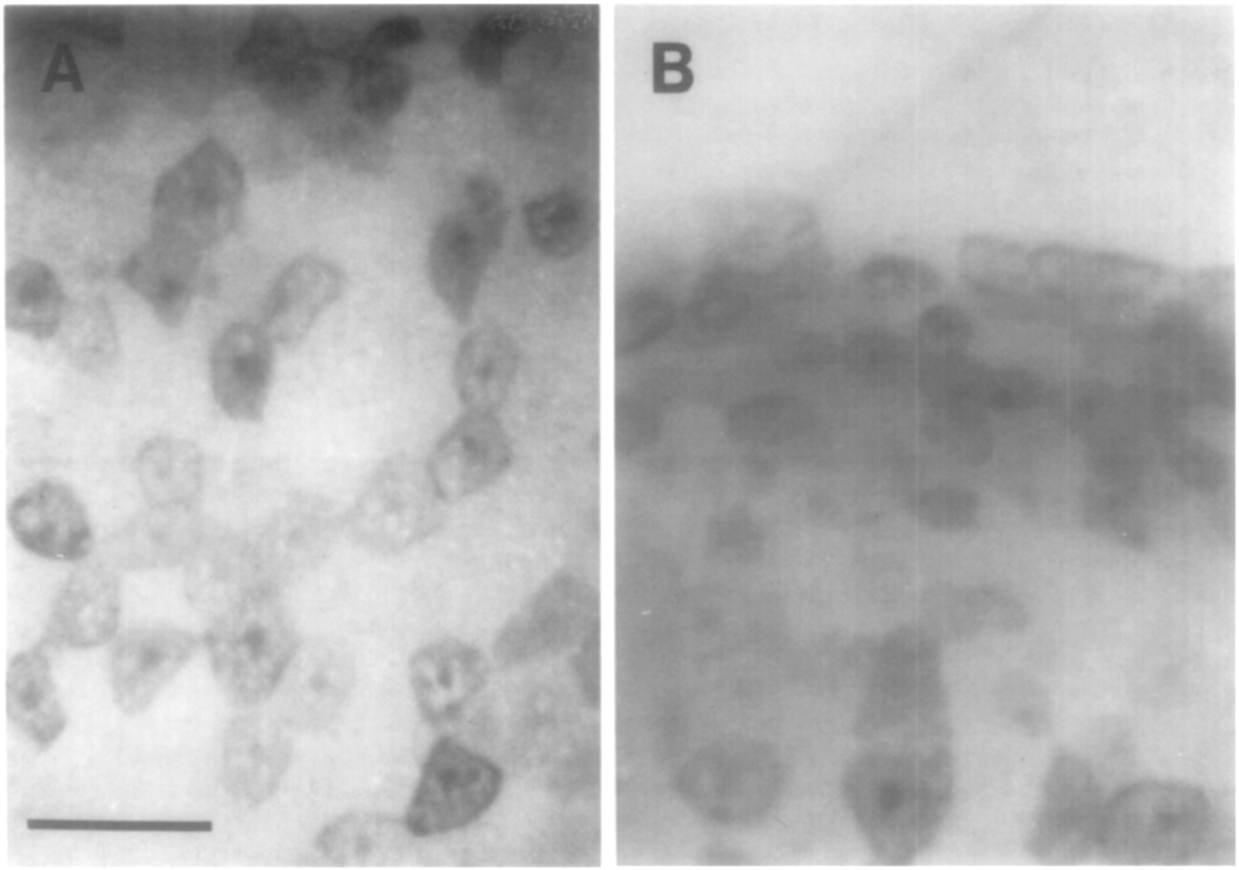


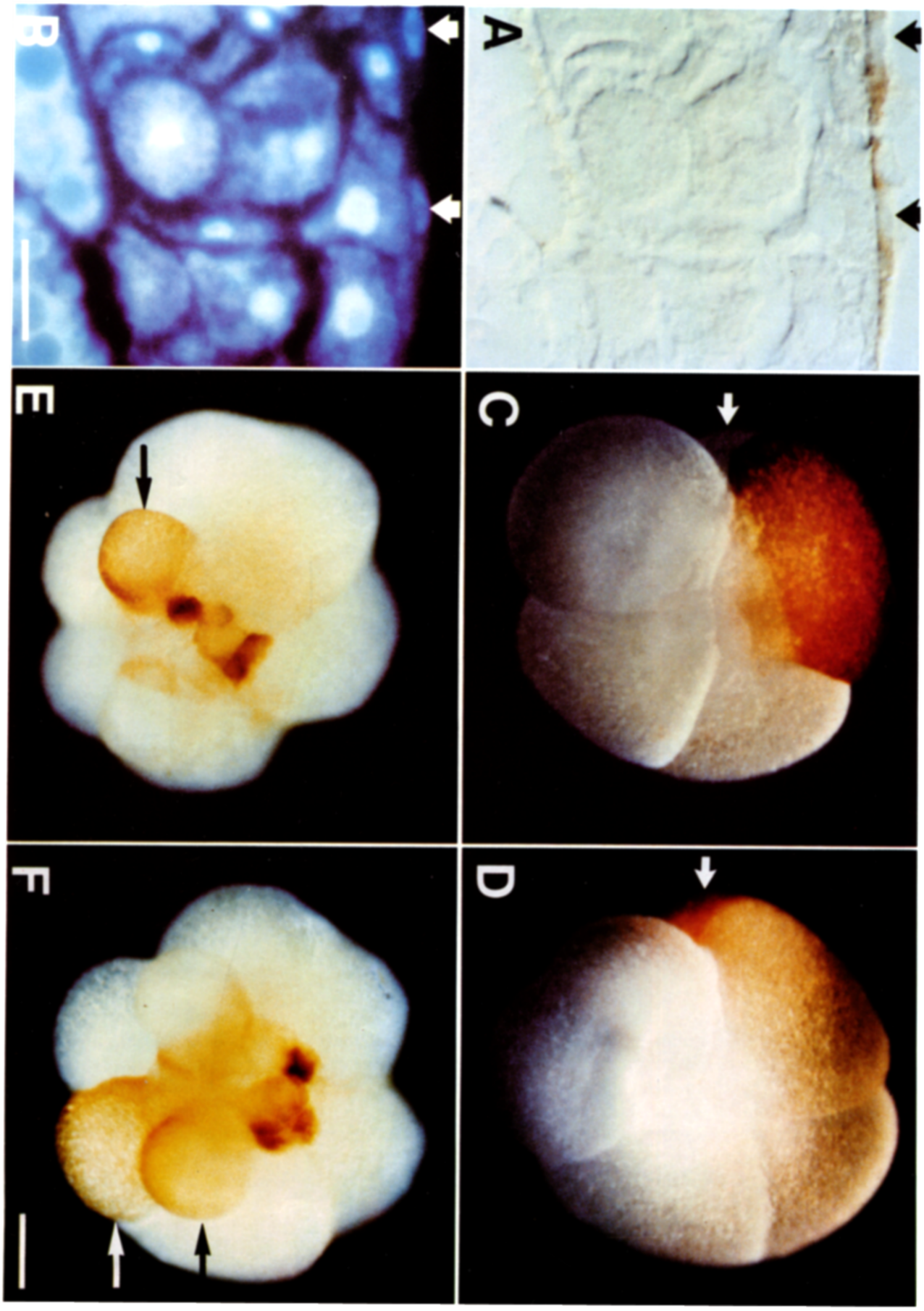
FIG. 6. *htr-wnt-A* expression in provisional epithelium of *Placobdella*. Stage 8 embryos were fixed and processed to immunolocalize *htr-wnt-A* protein. (A) Photomicrograph of a portion of the provisional epithelium showing the tile-like array of cells above the underlying macromere. (B) Photomicrograph showing the margin of the epithelium; note that the epithelial cells lying over the germinal bands (oriented horizontally across the center of the photograph) have significantly smaller apical areas. Scale bar, 100 μm.

development (Sandig and Dohle, 1988; Bissen and Weisblat, 1989). Of these, 9 arise via three rounds of division from the A, B, and C cells while the remainder come from the D cell, which also generates the teloblasts. Among the 16 micromeres descended from the D cell, 10 arise in bilateral pairs and the remaining 6 are unpaired.

Each teloblast undergoes several dozen highly unequal divisions to produce a coherent column, or *bandlet* of progeny called *primary blast cells* (stages 6-8). On each side, the bandlets come together in parallel arrays called *germinal bands* (stage 7). The left and right germinal bands are in contact with each other via their distal ends at the future head of the embryo and are separated from each other elsewhere by an epithelium derived from the cells of the micromere cap. This micromere-derived epithelium also covers the germ bands themselves.

As more blast cells are budded off by the teloblasts, the germinal bands lengthen and move across the surface of the macromeres gradually coalescing progressively from anterior to posterior aiong the future ventral midline into a structure called the *germinal plate* (stage 8). The micromere-derived epithelium expands

FIG. 7. Subcellular distribution of *htr-wnt-A* in normal embryos (A, B) and its distribution in aberrant embryos (C-F). (A, B) 3-μm transverse section through a germinal band of a mid stage 8 *Helobdella triserialis* embryo stained for *htr-wnt-A*, visualized with DAB and for DNA (Hoechst 33258). Apical surface of the epithelium is up. (A) Photomicrograph of the section viewed using DIC optics; the brown HRP reaction product in the squamous epithelium is excluded from nuclei (arrows) which are visible (B) when the same section is viewed with epifluorescence. (C-F) Aberrant embryos fixed and stained for *htr-wnt-A*. (C) A stage 4a embryo in which the A′ macromere is stained and (D) one in which the DM macromere is stained. (E) A stage 6a embryo in which the left N teloblast is stained and (F) an embryo in which both the right N teloblast and the right OPQ macromere are stained. Scale bar, 10 μm in A and B; 100 μm in C-F.


B
A
E
C
F
D

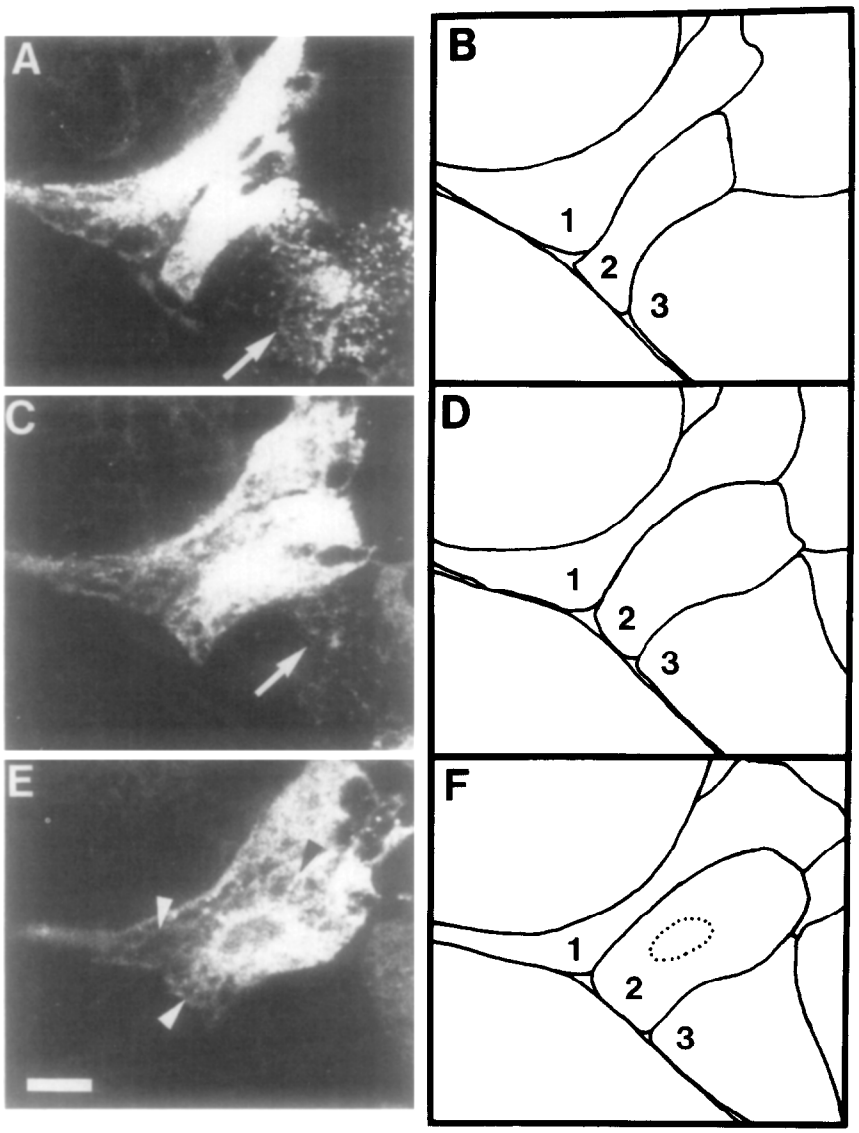


FIG. 8. Subcellular distribution of *htr-wnt-A*. A stage 5 *Helobdella triserialis* embryo fluorescently stained for *htr-wnt-A* using a fluorescent second antibody was viewed with a confocal microscope. (A, C, E) Three serial optical sections, each approximately 2-μm thick, taken at 7-μm intervals through three micromeres [designated as cells 1, 2 and 3 in the adjacent tracings (B, D, F)], adjacent to macromere C (lower left) and the right-hand NOPQ cell (upper left). (A, C) The arrows point to the punctate distribution of *htr-wnt-A* antigen associated with cell 3 and illustrates that it is predominantly localized at the plasma membrane as can be seen in the most superficial section (A, B). (E, F) The arrowheads indicate the reticulate distribution of *htr-wnt-A* antigen in the cytoplasm of cells 1 and 2; *htr-wnt-A* is concentrated around, but absent from, the nucleus (dotted circle in cell 2). Scale bar, 50 μm.

concomitantly, continuing to cover the germ bands and the area behind them with a squamous epithelium. Beneath the micromere-derived epithelium in the area between the bandlets lie muscle fibers of mesodermal (M teloblast) origin (Weisblat *et al.*, 1984). Together these two sets of cells constitute the *provisional integument*, which serves as a temporary body wall of the embryo, pending the generation of definitive body wall by the proliferation of cells in the germinal plate. All the segmental tissues of the leech arise from the proliferation and differentiation of cells within the germinal plate (stages 9–10), the lateral edges of which gradually expand and eventually meet at the dorsal midline (stage 10), forming the tube that makes up the body of the animal. As the germinal plate expands, the provisional integument retracts and is finally lost at the end of stage 10. Nevertheless, micromeres do contribute some definitive progeny to the leech, including nonsegmental tissues such as the neurons of the supraesophageal ganglion, the epidermis of the prostomium (Weisblat *et al.*, 1984), and cells in the proboscis (F. Ramirez, personal communication).

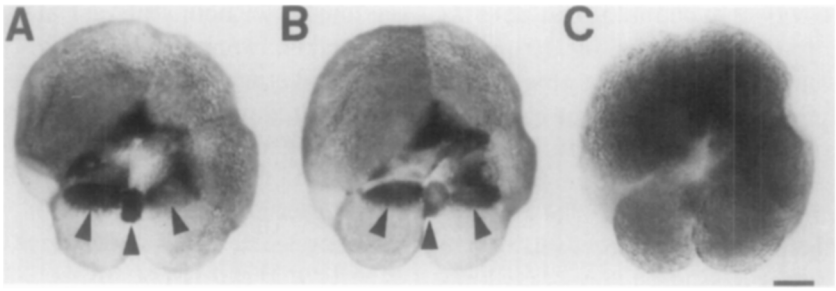


FIG. 9. Nonradioactive *in situ* localization of *htr-wnt-A* transcripts. Very early stage 5 embryos of *Helobdella robusta* hybridized with antisense (A and B) and sense probe (C) for *htr-wnt-A* transcripts. Cells NOPQ left and right (bottom) are just forming and micromeres dnopq″, dnopq‴, and dnopq′ are stained in embryos hybridized with antisense probe (black arrowheads). Cells dnopq′ and dnopq″ also stained immunohistochemically after stage 5 embryos were incubated with anti-*htr-wnt-A* antibodies (see Figs. 4D–4F). Scale bar, 100 μm.

Isolation of htr-wnt-A

Sequence information for *Wnt*-1, *Wnt*-2, and *wingless* was used to identify regions of conserved protein sequence and low codon degeneracy to which complementary DNA oligonucleotide primers might be designed for use in the PCR. To lessen the chance that intron sequences would interfere with PCR amplifications of leech genomic DNA, we took into account the evolutionarily conserved intron-exon structure of *Wnt*-1 (van Ooyen and Nusse, 1984; van Ooyen *et al.*, 1985) and *wingless* (Rijsewijk *et al.*, 1987) and chose to amplify sequences wholely contained within the third exon of the *Drosophila* and mouse genes (Fig. 2A).

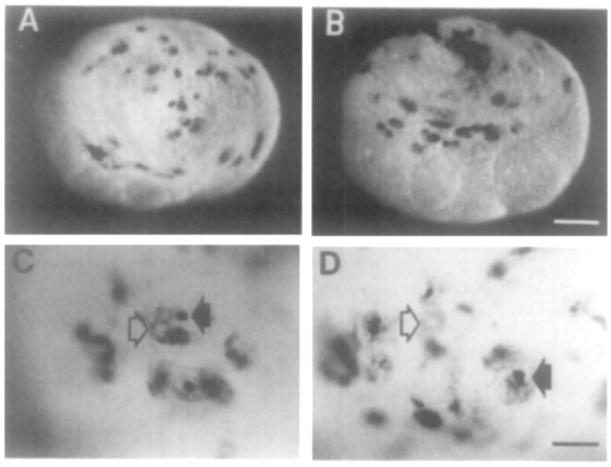


FIG. 10. Nonradioactive *in situ* localization of *htr-wnt-A* transcripts. Early stage 8 embryos of *Helobdella triserialis* hybridized for *htr-wnt-A* transcripts. (A) View of the animal pole; the entire provisional epithelium can be seen (compare with Fig. 5G–5I). (B) Another embryo, viewed from the prospective caudal end, shows the unstained teloblasts (bottom). (C, D) Higher magnification views of groups of hybridizing cells of the provisional epithelium. Solid arrows indicate cells with substantial nuclear and/or perinuclear hybridization; open arrows indicate cells with primarily diffuse cytoplasmic hybridization and little or no nuclear hybridization. Scale bar, 100 μm in A and B; 20 μm in C and D.

PCR amplification of genomic DNA from *H. triserialis* yielded DNA fragments of the size expected for *Wnt* family members. The fragments were inserted into a plasmid vector. DNA sequence analysis revealed five of five clones contained the same amplified sequence and that this sequence had the capacity to encode a protein with sequence similarity to the *Wnt* class of proteins (Fig. 2A).

A plasmid clone of the amplification product was used to screen a *H. triserialis* genomic DNA λ library, and positive λ phage clones were obtained. The restriction maps of these positive clones overlapped extensively, indicating that they were all from the same genomic region. The chromosomal DNA sequences of one of the phage positives was subcloned into a plasmid vector and partially sequenced. The nucleotide sequence obtained (Fig. 2B) overlaps and extends the sequence obtained from the PCR product. Comparison of the 87 amino acid predicted translation product with those of other *Wnt* class genes (Fig. 2B) reveals that the 86 amino acid exon of *htr-wnt-A* shares 49 amino acid identities (57%) with *Wnt*-2, 36 (42%) with *Wnt*-1, and 39 (45%) with *wingless*.

Analysis of htr-wnt-A Protein Distribution

To obtain antibodies directed against the *htr-wnt-A* protein, we first constructed bacterial expression vectors to facilitate the production and purification of sufficient quantities of *htr-wnt-A* antigen. Since our goal was to raise antibodies against a relatively small portion of the protein, we sought to enhance its immunogenicity by designing a bacterial expression plasmid that would direct the synthesis of a fusion protein between *E. coli* β-galactosidase and 16 tandem repeats of the 83 amino acid fragment of the *htr-wnt-A* protein (BG16xhtr-wnt-A) shown in Fig. 2B. Thus, a 260-kDa fusion protein, approximately half of which was *htr-wnt-A*, was expressed, purified, and used as an immunogen. The resultant antisera was affinity purified em-

ploying a column with covalently bound *E. coli* anthranilate synthase fused to four repeats of *htr-wnt-A* (AS4xhtr-wnt-A). We reasoned that use of this fusion protein would have the effect of increasing the column's capacity for *htr-wnt-A* antibodies relative to its capacity for unwanted antibodies that might fortuitously cross-react with *E. coli* anthranilate synthetase. The affinity-purified *htr-wnt-A* antibodies showed no reactivity to protein blots of crude lysates of *E. coli* expressing β-galactosidase or anthranilate synthase, but did cross react to *E. coli*-expressed *htr-wnt-A* protein (data not shown). On immunoblots, the purified anti-*htr-wnt-A* antibody preparation identifies a single band corresponding to a protein species of molecular weight 32,000 daltons (Fig. 3).

The spatial and temporal pattern of *htr-wnt-A* expression was determined using the antibodies generated against the *htr-wnt-A* coding region. Leech embryos at various stages of development were fixed, reacted with anti-*htr-wnt-A* antiserum, and visualized with either a fluorescein- or an HRP-labeled second antibody. Fluorescein-labeled embryos were examined by confocal microscopy, while the histochemically processed HRP-labeled embryos were either viewed in whole-mount or sectioned and viewed with DIC optics. Control immunolocalization experiments which omitted anti-*htr-wnt-A* antiserum were devoid of signal (data not shown). While the exact pattern of *htr-wnt-A* expression during early embryogenesis varies noticeably from embryo to embryo, an underlying pattern can be discerned in that expression is restricted to micromeres and epithelial cells in the provisional integument.

htr-wnt-A expression is first detectable at the 8-cell stage (stage 4a) in micromere d′ a member of the primary quartet (Figs. 4A–4C). Expression is seen in only half the embryos at this stage; the remaining embryos have no detectable expression in any cell.

At early stage 5, more than 90% of the embryos express *htr-wnt-A* in the dnopq′ and dnopq″ micromeres, while other micromeres exhibit more embryo to embryo variability in their *htr-wnt-A* expression state (Figs. 4D–4F). All embryos have at least 2 *htr-wnt-A* expressing and at least 2 *htr-wnt-A* nonexpressing micromeres of the 15 micromeres present at this stage. The relative levels of expression in different cells also vary between embryos.

In stage 6a embryos, the pattern of *htr-wnt-A* expression shows even greater variability than during earlier stages (Figs. 4G–4I). During stages 5 and 6 additional micromeres are produced both from macromeres and by proliferation of micromeres that were born earlier (F. Ramirez, personal communication). These factors make it impossible to identify individual micromeres unambiguously without the assistance of lineage tracers (Kostriken, work in progress).

By mid stage 7, the squamous epithelium of the provisional integument has formed from some of the micromere progeny. At this time, *htr-wnt-A* expression is restricted to cells within the provisional epithelium, but the expression pattern is a patchwork of expressing and nonexpressing cells that varies extensively from embryo to embryo (Figs. 5A–5C). Identically staged embryos stained with silver to demarcate the cells of the provisional epithelium (Figs. 5D–5F) confirm previous observations that the pattern of cells comprising the epithelium is also variable (Ho and Weisblat, 1987).

During stage 8, the provisional epithelium undergoes an epibolic expansion, continuing to cover the germ bands and the area behind them as they move across the surface of the embryo and coalesce into the germinal plate (Figs. 5G–5I and 5M–5O). Much of the epithelium is a single cell deep and the surface layer can be visualized by silver staining (Figs. 5J–5L and 5P–5R). Approximately 25% of the cells stain positively for *htr-wnt-A* antigen. This fraction is not noticeably different in embryos fixed at different times during stage 8. As in stage 7, the precise pattern of staining varies from embryo to embryo (Figs. 5G–5I and 5M–5O).

Species Cross Reactivity of the Anti-htr-wnt-A Antibodies

To examine the cross-reactivity of the anti-*htr-wnt-A* antibodies, stage 8 embryos from several other leech species were immunostained. The antibodies cross-react well with all species examined, including *H. robusta, H. stagnalis, Theromyzon rude,* and *Placobdella sp.* In each of these species, *htr-wnt-A* is expressed in cells of the provisional epithelium during stage 8 in patterns similar to those observed for *H. triserialis.* The salt-and-pepper quality of the pattern of *htr-wnt-A* expression is especially clear in *Placobdella,* because the provisional epithelium of these embryos contains many more cells than *Helobdella* embryos at a comparable stage (Figs. 6A and 6B). As in *Helobdella,* the precise distribution of *htr-wnt-A* expressing cells and their individual levels of *htr-wnt-A* expression show no reproducible pattern from embryo to embryo.

Subcellular Localization of the htr-wnt-A protein

In many whole-mount preparations observed under the dissecting microscope, *htr-wnt-A* antigen appeared to be concentrated in the center of the cell near the nucleus. To obtain subcellular resolution of *htr-wnt-A* expression, transverse sections were made through immunohistochemically processed stage 8 embryos for examination under the compound microscope (Figs. 7A and 7B). In epithelial cells overlying the blast cells in the

germinal band, *htr-wnt-A* antigen can be seen throughout the cytoplasm but is excluded from the nucleus, consistent with protein transit through the perinuclear endoplasmic reticulum, as expected for a secreted protein. Fluorescently labeled secondary antibodies in conjunction with confocal microscopy gave a higher resolution picture of the subcellular distribution of *htr-wnt-A* (Fig. 8); using this technique, *htr-wnt-A* was seen at the cell surface and in the cytoplasm. The *htr-wnt-A* antigen occurs in a reticulate pattern radiating from the nuclear periphery in both the apical and basal directions toward the cell surface, as expected for a protein that traverses the cytoplasm by way of membranous compartments (Figs. 8E and 8F). Other cells display a punctate pattern of *htr-wnt-A* antigen concentrated primarily at the cell surface (Figs. 8A–8D). A similar punctate distribution has been noted for the *wingless* antigen in *Drosophila* embryos (van den Heuvel *et al.*, 1989).

Analysis of htr-wnt-A Transcription

Using strand-specific digoxygenin-labeled hybridization probes generated from plasmid clones of *htr-wnt-A*, we performed *in situ* hybridizations to fixed stage 5 *H. robusta* and stage 8 *H. triserialis* embryos. Histochemical visualization of the hybridized probe using alkaline phosphatase-conjugated, anti-digoxygenin antibodies revealed the distribution of complementary embryonic RNA.

The hybridization pattern of probes made from the 260 base pair coding region described in Fig. 2 to stage 5 embryos is shown in Figs. 9A–9C. Hybridization of the antisense probe (Figs. 9A and 9B) identified a subset of micromeres, including the three unambiguously identified micromeres (dnopq′, dnopq″, and dnopq‴) derived from cell DNOPQ (see Fig. 1). As expected, this staining pattern was strongly reminiscent of that obtained with anti-*htr-wnt-A* antiserum on stage 5 embryos (Figs. 4D–4F), differing mainly in that the last-born of these cells, micromere dnopq‴, had accumulated RNA but had not yet accumulated detectable levels of protein by stage 5. The sense probe (Fig. 9C) did not hybridize to any cells in the embryos.

Figures 10A and 10B shows stage 8 embryos visualized for *htr-wnt-A* transcripts using an antisence probe made to a 4-kb region of genomic DNA encompassing the *htr-wnt-A* coding region described in Fig. 2; the intracellular distribution of the transcript is shown at higher magnification in panels C and D. As with its translation product the *htr-wnt-A* transcript was restricted to a subset of cells (approximately 25%) within the provisional epithelium. No transcripts were detected in teloblasts or blast cells. The similarities between the *htr-wnt-A* transcript and protein distribution suggest that the *htr-wnt-A* antigen is found predominantly in association with the cells producing it rather than in nonexpressing cells that accumulate it.

Within the epithelial cells that bound the *in situ* hybridization probe, we observed variability in both the subcellular localization and the intensity of the reaction product (Figs. 10C and 10D). Approximately 75% of the cells that were positive for *htr-wnt-A* (172 of 225 randomly surveyed from stage 8 embryos) showed hybridization predominantly over the nucleus or over both the nucleus and the cytoplasm, as if they had either only recently initiated *htr-wnt-A* transcription or had achieved a steady-state distribution of transcripts in the nucleus and/or in the closely apposed endoplasmic reticulum, as would be expected of a protein that enters the secretory pathway. The remaining *htr-wnt-A* mRNA-positive cells showed weak hybridization predominantly in the cytoplasm as if transcription had ceased and only residual *htr-wnt-A* mRNA remained. In light of this apparently dynamic transcription pattern and the roughly equivalent numbers of cells transcribing and translating *htr-wnt-A*, we suggest: (1) that different cells within the epithelium are continually initiating and terminating *htr-wnt-A* transcription during these stages and (2) that cell-associated *htr-wnt-A* protein is short-lived, so that antigen disappears relatively soon after its messenger RNA turns over.

Unusual htr-wnt-A Expression Patterns

While the details of the patterns of *htr-wnt-A* expression vary from embryo to embryo, the observed patterns were uniform in that normally only micromeres and, in later embryos, provisional epithelial cells stained in normal embryos; occasional exceptions to this rule were observed however among embryos drawn from aberrant clutches (see Materials and Methods). In 10–30% of the embryos from these clutches, one or more teloblasts, teloblast precursors, or macromeres expressed *htr-wnt-A;* even outwardly normal embryos from the aberant clutches showed this ectopic expression (Figs. 7C–7F). In some embryos the ectopic expression appeared to be at the expense of the normal expression in micromeres (Figs. 7C and 7D), while in others, the ectopic expression was in addition to apparently normal expression within micromeres (Figs. 7E and 7F).

DISCUSSION

The *Wnt* gene family codes for secreted proteins, at least some of which are known to be important in mediating cell–cell interactions during embryogenesis. In the experiments reported here, we have cloned a fragment of a *Wnt* family member from an annelid, the leech *H. triserialis,* and employed a novel iteration procedure

to generate antibodies to a relatively small portion of this protein, *htr-wnt-A*. Using these antibodies and transcript-specific *in situ* probes, we have studied the transcription and translation of *htr-wnt-A* during embryogenesis. Our results indicate that *htr-wnt-A* is expressed in somewhat sterotyped patterns by micromeres during stages 4–6 and then in an apparently dynamic and nonstereotyped manner by cells of a transient embryonic epithelium during stages 7–8.

Homology of htr-wnt-A to Other Wnt Genes

All clones sequenced from both the original PCR amplification and the genomic library screen were identical. Amino acid sequence homology is strictly limited to the region we anticipated to be a phylogenetically conserved exon. Outside this region, the DNA sequence is low in G+C content, characteristic of untranslated introns. Therefore, both the protein coding capacity and the putative intron-exon arrangement of this leech gene are homologous to other members of the *Wnt* gene family. In addition the molecular weight of *htr-wnt-A*, as estimated from immunoblots (Fig. 3), is consistent with a *Wnt* class protein. The predicted amino acid sequence of this portion of *htr-wnt-A* shares significant homology with comparable portions of both the vertebrate and insect *Wnt* proteins. In particular, on the basis on the 86 amino acids in the putative exon we have characterized, *htr-wnt-A* is most similar to *Wnt*-2 and is much less similar to *Wnt*-1 (mouse) or *wingless* (fly). Thus, while there is no evidence from this work of more than one *Wnt* gene family member in leech, it seems likely that at least one other *Wnt* homolog will be found and that it will be as homologous to *wingless* and *Wnt*-1 as these two gene products are to each other. One unusual feature of the *htr-wnt-A* coding region is a cysteine at amino acid 58 not found in the homologous position of other *Wnt* proteins (Fig. 2). Participation of this cysteine in a disulfide linkage might set *htr-wnt-A* apart structurally from other *Wnt* proteins.

Variability in the Pattern of htr-wnt-A Expression

While the exact pattern of *htr-wnt-A* expression varies from embryo to embryo through all stages of development, it is significantly more stereotyped earlier in development than later. During the earliest expressing stage (stage 4a), *htr-wnt-A* is strictly confined to micromere d′ and the only variability we observed was in whether or not cell d′ expressed *htr-wnt-A* at detectable levels. Through stage 6, certain micromeres tend to express the *htr-wnt-A* protein but there is also noticeable variability between embryos as to precisely which micromeres express *htr-wnt-A*, as well as the relative levels of expression (Fig. 4).

Once the embryos have reached stage 7, most or all of the initial 25 micromeres have contributed progeny to the squamous epithelium of the provisional integument. The precise cellular organization of the epithelium does not appear to be stereotyped with respect to cell number or division pattern which makes comparisons among embryos problematic. The distribution of *htr-wnt-A* expressing cells during these stages is variable between embryos with respect to the number of cells and their position within the provisional epithelium, and the pattern is not bilaterally symmetric. Since at this point we can no longer identify individual cells in the epithelium we cannot completely exclude the possibility that *htr-wnt-A* expression is confined to particular sublineages of cells. However, this seems very unlikely given the variability in expression by identified cells seen in stage 6 embryos. In any case, by comparing the salt-and-pepper expression patterns observed here with the coherent clones of epithelial cells derived from individual micromeres at this stage (Ho and Weisblat, 1987), we *can* be sure that (1) not all the descendants of a given micromere express *htr-wnt-A* continuously throughout embryonic development and (2) one or more descendants of all or almost all micromeres *do* express *htr-wnt-A* at some time(s) during stages 7–8.

This apparently stochastic pattern of *htr-wnt-A* expression among micromeres and their descendants persists throughout many hours of embryonic development without a profound change in the proportion of cells expressing the gene. Thus, the observed heterogeneity of expression does not simply result from an asynchronous transition of cells within the epithelium from a state of uniform nonexpression to a state of uniform expression.

Two possible mechanisms to account for the observed pattern of *htr-wnt-A* expression are as follows:

(1) Although the cells of the provisional epithelium that stain for *htr-wnt-A* are otherwise indistinguishable from those that do not, it may be that there are in fact two (or more) intrinsically distinct classes of cells, only one of which expresses *htr-wnt-A*. The notion that the cells of the provisional epithelium, previously assumed to be homogeneous, are in reality composed of at least two cell types, has precedents in other embryos. In *Drosophila*, for example, patterned expression of zygotic genes within the morphologically homogeneous cellular blastoderm distinguish many different classes of cells destined for distinct developmental fates (for a review see Akam, 1987). And more recently, it has been reported that a diffusely distributed subpopulation of cells within the epiblast of the chick embryo stains with the HNK-1 monoclonal antibody and that these cells are destined for the primitive streak (Stern and Canning, 1990). However, in contrast to these examples from *Dro-*

sophila and chick, all the cells in the leech provisional epithelium appear to have the same fate.

(2) Alternatively, it may be that all cells in the provisional epithelium can express *htr-wnt-A* and the mottled expression pattern is a consequence of the fact that *htr-wnt-A* expression by any given cell is transient. Our *in situ* analysis of *htr-wnt-A* transcripts supports the notion that individual provisional epithelial cells initiate and terminate *htr-wnt-A* transcription during stage 8. Whether all cells, or merely a specific subset of the provisional epithelium undergo such fluctuations remains to be determined. Since the cells of the provisional epithelium are not in mitotic synchrony a dynamic expression pattern would arise if expression is limited to a small part of the cell cycle.

Role of htr-wnt-A in Embryonic Development

Wnt gene family members are known to be communication molecules, in some instances functioning as critical signaling molecules during embryogenesis (Rijsewijk *et al.*, 1987; McMahon and Bradley, 1990; Thomas and Capecchi, 1990). The effect exerted by *Wnt* molecules may occur among expressing cells (Brown *et al.*, 1986) or on adjacent cells that are not expressing (van den Heuvel *et al.*, 1989). Our experiments reveal that *htr-wnt-A* remains localized to the immediate vicinity of the cell expressing it, consistent with other experiments showing that cultured cells engineered to express *Wnt*-1 fail to excrete freely diffusible *Wnt*-1 gene product (Papkoff and Schryver, 1990). From these observations, we expect that the direct effects exerted by *htr-wnt-A* would be limited to the immediate neighbors of cells expressing the protein.

A recent analysis of transcription in leech embryos (Bissen and Weisblat, 1991) showed that the earliest zygotic mRNA transcription begins at stage 5, as detected autoradiographically by α-amanitin sensitive UTP incorporation. Here, using specific and sensitive probes for *htr-wnt-A*, we detect zygotic gene expression even earlier, during stage 4a. We also find that *htr-wnt-A* expression in *H. triserialis* is sensitive to α-amanitin (our unpublished results). Hence, we conclude that *htr-wnt-A* is among the earliest zygotic transcripts in *H. triserialis*. At present we can only speculate on the function of *htr-wnt-A*, since we are not yet able to selectively eliminate the protein from the embryo. But the earliest effects of α-amanitin poisoning in *Helobdella* are altered cleavages during teloblast formation between stages 4 and 6 (Bissen and Weisblat, 1991). Thus, one possible role for *htr-wnt-A* is in cell-cell communications regulating the stereotyped set of cleavages during teloblast formation. The observation that *htr-wnt-A* is expressed ectopically in embryos from aberrant clutches is consistent with this hypothesis, but certainly does not prove it, since the data on ectopic expression are merely correlative.

During stages 7–8 embryonic epithelial cells expressing *htr-wnt-A* are in contact with several cell types, including macromeres, other epithelial cells, nonepithelial micromere derivatives, teloblasts, and the blast cells of the underlying ectodermal (n, o, p, and q) bandlets, precursors of the segmental ectoderm. Thus, *htr-wnt-A* may mediate interactions between any or all of these cell types. For example, previous experiments have shown that the overlying provisional epithelium is essential for normal fate-determining interactions with the equivalence group composed of o and p blast cells (Ho and Weisblat, 1987). Thus, one role for *htr-wnt-A* may be to regulate cell fates in ectodermal cell lineages by mediating interactions of the O-P equivalence group (Shankland and Weisblat, 1984).

Homologies of htr-wnt-A and Other Wnt Genes

Segments in annelids and arthropods are held to be homologous structures both on classical taxonomic grounds and on the basis of more recent molecular analyses of *engrailed* gene expression (Wedeen and Weisblat, 1991). It is thus of interest to compare *Wnt* gene expression in *Helobdella* and *Drosophila* in light of what is known about both the processes of segmentation and of *engrailed* expression.

Wingless, the *Drosophila Wnt*-1 homolog, is required for normal development of segments and is initially expressed in a series of segmentally iterated rows of cells shortly after cellular blastoderm formation, just anterior and adjacent to the rows of *engrailed* expressing cells (Baker, 1987; van den Heuvel *et al.*, 1989). In the absence of the *wingless* function, *engrailed* expression initiates normally but disappears prematurely (DiNardo *et al.*, 1988; Martinez-Arias *et al.*, 1988) resulting in embryos lacking posterior compartments of their segments. Thus, *engrailed* expressing cells require the extracellular signal, *wingless*, supplied by neighboring cells in order to achieve posterior compartment cell fates, i.e., stable *engrailed* expression (Heemskerk *et al.*, 1991).

The leech *engrailed* homolog is also expressed in segmentally iterated rows of cells in the germinal plate, i.e., in cells that contribute directly to definitive segmental tissues and its expression pattern exhibits other parallels to that seen in *Drosophila* as well (Wedeen and Weisblat, 1991). Thus, the fact that *htr-wnt-A* is expressed exclusively in cells that do not contribute to segmental tissues in leech and that the spacial pattern of expression lacks the consistancy expected of a gene that regulates pattern formation, argues against the notion

that *htr-wnt-A* might play a role in segmentation homologous to that played by *wingless*. Rather we expect that there are multiple *Wnt* genes in leech as in other organisms, and that one of these as yet undiscovered genes is at least as homologous, on the level of amino acid sequence, to *wingless* as is *Wnt*-1; whether or not such a leech *Wnt*-1 homolog participates either in the process of segmentation or *engrailed* regulation is a question that would bear on the evolutionary relationship of annelid and arthropod segmentation.

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