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Expression of Actin Genes in the Arrow Worm *Paraspadella gotoi* (Chaetognatha)

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ABSTRACT—Arrow worms (the phylum Chaetognatha), one of the major marine planktonic animals, exhibit features characteristic to both deuterostomes and protostomes, and their ancestry therefore remains unknown. As the first step to elucidate the molecular bases of arrow worm phylogeny, physiology and embryology, we isolated cDNA clones for three different actin genes (*PgAct1*, *PgAct2* and *PgAct3*) from the benthic species *Paraspadella gotoi*, and examined their expression patterns in adults and juveniles. The amino acid sequences of the three actins resembled each other, with identities ranging from 86% to 92%. However, the patterns of the spatial expression of the genes were independent. The *PgAct1* gene might encode a cytoplasmic actin and was expressed in oogenic cells, spermatogenic cells, and cells in the ventral ganglion. The *PgAct2* and *PgAct3* genes encoded actins of divergent types. The former was expressed in well-developed muscle of the head (gnathic) region and trunk muscle cells, whereas the latter was expressed in muscle of the trunk and tail regions and oogenic cells. These results suggest that, similarly to other metazoans, the chaetognath contains multiple forms of actins, which are expressed in various manners in the adult and juvenile arrow worm.

INTRODUCTION

Arrow worms (the phylum Chaetognatha) are about 100 species of marine, largely planktonic animals, with the exception of a few benthic species (cf., Bone *et al.*, 1991). According to Brusca and Brusca (1990), they are bilateral deuterostomes, and are characterized by features including a trimeric body comprised of a head, trunk and postanal tail divided from one another by transverse septa, a body with lateral and caudal fins, a head with a pair of uniquely arranged eyes, and, around the mouth, sets of grasping spines and teeth used for prey capture. Arrow worms possess longitudinal muscles of an unusual type, arranged in quadrants rather than a circular arrangement, a complete gut anus at the ventral surface of the trunk-tail junction, and a central nervous system including a cerebral ganglion in the dorsal side of the head and a large ventral ganglion in the trunk. These animals are hermaphroditic and direct developers.

The phylogenetic status of chaetognaths is mysterious. They share some common characteristics with deuterostomes during their ontogeny; radial cleavage, a blastopore at the rear end of the body, and a postanal tail (Hyman, 1959; Brusca and Brusca, 1990; Willmer, 1990). However, the morphology of adults - namely, a coelom without a peritoneum and the apparent lack of circular muscle in the body wall - suggests their similarity to pseudocoelomate groups (Willmer, 1990), although their coelomic condition is still debated (Shinn and Roberts, 1994). Their nervous system is more like that of protostomes (Rehkämper and Welsch, 1985; Goto and Yoshida, 1987). Moreover, even during ontogeny, they do not pass through the dipleurula stage that is seen in a few deuterostome phyla. Recent molecular phylogenetic studies using 18S rDNA (rRNA) sequences did not support the affinity of chaetognaths with deuterostomes (Telford and Holland, 1993; Wada and Satoh, 1994).

Despite such characteristics and their phylogenic position, arrow worms have been a subject of very few molecular biological investigations. Our laboratory at the Mie University has recently succeeded in the maintenance of the benthic species *Paraspadella gotoi* by extending several generations. *P. gotoi* may provide an appropriate experimental system to investigate the molecular bases of the physiology, behavioral biology and developmental biology of the arrow worm. As the first step to elucidate the molecule basis, the present study was performed to isolate cDNA clones for actin genes from *P. gotoi*. Actin is a ubiquitous protein that is encoded by a multigene family in a variety of animals (Pollard and Cooper 1986; Rubenstein, 1990). There are two subtypes of actin muscle type and cytoplasmic type (Vandekerckhove and

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Weber, 1984). Several kinds of muscle-type actin mRNA have also been shown in certain higher eukaryotes. The coding sequences of different members of the actin gene family are conserved, whereas the 5' and 3' non-coding sequences usually diverge and can be used as gene-specific probes (Shani *et al.*, 1981). Each actin isoform shows a distinct expression pattern specific to tissues or developmental stages (Sanchez *et al.*, 1983; Schwartz and Rothblum, 1981; Kusakabe *et al.*, 1995). The present study revealed that the arrow worm also contains multiple forms of actin, which show different patterns of spatial expression.

MATERIALS AND METHODS

Animals

Paraspadella gotoi Casanova (Casanova, 1990) was collected in the vicinity of Amakusa Marine Biological Station, Kyushu University, Kumamoto, Japan. *P. gotoi* belongs to the order Pharagmorpha with ventral transverse muscle bands (pharagma) and is a benthic species. Adults specimens have been maintained in our laboratory at Mie University in a constant-temperature room at 17°C and fed with *Tigriopus japonicus*. They are cross-fertile and fertilization occurs internally. Juveniles were obtained by collecting laid eggs and were kept in a constant-temperature room at 23°C. The young hatched 2 days after egg-laying. Details of the culture method have been described elsewhere (Goto and Yoshida, 1997).

Isolation of RNA and construction of a cDNA library of *P. gotoi* adult

Total RNA was isolated from whole adult specimens (about 300 individuals) by the acid guanidinium thiocyanate-phenol-chloroform (AGPC) method (Chomczynski and Sacchi, 1987). Poly(A)$^+$ RNA was purified by use of Oligotex-dT30 Latex beads (Roche Japan, Tokyo) according to the manufacturer's protocol. Complementary DNA was synthesized from the poly(A)$^+$ RNA with a Zap cDNA Synthesis kit (Stratagene, La Jolla, CA, USA). Double-stranded cDNA was size-fractionated on a column of Sephacryl S-500 (Pharmacia Biotech, Uppsala, Sweden), and fractions that contained fragments more than 300 bp in length were collected. The double-stranded cDNA was cloned directly into the *Eco*RI-*Xho*I site of a Uni-ZAPXR vector (Stratagene). The titer of the amplified cDNA library was estimated to be 2.4×10^7 pfu/μl.

Isolation and sequencing of cDNA clones for *P. gotoi* actin genes

The amino acid sequences of actins are highly conserved among eukaryotes (e.g., Kusakabe *et al.*, 1997). The primers were designed as follows: muscle forward, 5'-TG(C/T)GA(C/T)AA(C/T)GG(A/C/G/T)(A/T)(C/G)(A/C/G/T)GG(A/C/G/T)(C/T)T-3'; cytoplasmic forward, 5'-GT(A/C/G/T)GA(C/T)AA(C/T)GG(A/C/G/T)(A/T)(C/G)(A/C/G/T)GG(A/C/G/T)ATG-3'; and actin reverse, 5'-AA(A/G)CA(C/T)TT(A/C/G/T)C(G/T)(A/G)TG(A/C/G/T)AC(A/G/T)AT-3'. Using these oligonucleotide primers, we amplified target fragments from the first-stranded cDNAs which were obtained from RNAs of *P. gotoi* by means of reverse transcription-polymerase chain reaction (RT-PCR). Annealing was carried out at 37°C or at 42°C. Sequencing revealed that the amplified fragments were of actin genes.

Probing with candidate fragments random-labeled with [^{32}P]-dCTP (Amersham, Buckinghamshire, UK), we screened the cDNA library at moderate stringency conditions (hybridization; 5x SSPE, 0.5% SDS, 5x Denhardt's solution, 35% formamide at 42°C: washing; 2x SSC, 0.1% SDS at 37°C for 30 min twice and at 42°C for 30 min once: Sambrook *et al.*, 1989) and obtained many positive clones.

The nucleotide sequences were determined for both strands with a dye primer cycle sequencing FS ready kit and ABI PRISM 377 DNA sequencer (Perkin Elmer, Norwalk, CT, USA).

In situ hybridization

In situ hybridization was carried out with both whole-mount specimens of newly-hatched juveniles and sectioned specimens of adults. All specimens were fixed in 4% paraformaldehyde in MOPS buffer (pH 7.5), 0.5 M NaCl. Fixed specimens were immersed in 80% ethanol and kept at –20°C until use. The probes were synthesized from the 3' untranslated region of the gene by following the instructions from the supplier of the kit (DIG RNA Labeling kit; Boehringer Mannheim, Mannheim, Germany).

Whole-mount specimens: After a thorough washing with PBT [phosphate-buffered saline (PBS) containing 0.1% Tween 20], the fixed specimens were treated with 2 μg/ml proteinase K (Merck, Darmstadt, Germany) in PBS for 20 min at 37°C, and then they were post-fixed with 4% paraformaldehyde in PBS for 1 hr at room temperature. After a 1-hr period of prehybridization at 42°C, the specimens were allowed to hybridize with the digoxigenin-labeled antisense

```
1    GCACGAGAAAAGACGAGACGCGCTAACGGTTTTTCTGTCGAGAAAAAAAAATCCACTTTT  60
61   TAAACTTTTCTCTCCGTCGAATTTTTCGATCTTTTTAAAGCTCAAACAAACAAAATGTGC  120
                                                          M  C
121  GACGAAGAAGTTGCCGCTTTGGTCGTGGACAATGGTTCCGGAATGTGCAAGGCCGGTTTC  180
     D  E  E  V  A  A  L  V  V  D  N  G  S  G  M  C  K  A  G  F
181  GCCGGCGACGACGCCCCCGCGCCGTCTTCCCCTCCATCGTGGGACGCCCCCGTCATCAG  240
     A  G  D  D  A  P  R  A  V  F  P  S  I  V  G  R  P  R  H  Q
241  GGCGTCATGGTGGGCATGGGACAGAAGGACTCTTATGTGGGAGACGAGGCCCAATCCAAG  300
     G  V  M  V  G  M  G  Q  K  D  S  Y  V  G  D  E  A  Q  S  K
301  AGGGGCATCCTCACTCTGAAATACCCCATCGAACACGGCATCGTCACCAACTGGGACGAC  360
     R  G  I  L  T  L  K  Y  P  I  E  H  G  I  V  T  N  W  D  D
361  ATGGAGAAGATCTGGCATCACACCTTCTACAACGAGCTTCGCGTGGCGCCAGAGGAGCAT  420
     M  E  K  I  W  H  H  T  F  Y  N  E  L  R  V  A  P  E  E  H
421  CCCGTCCTGCTGACGGAGGCCCCTCTCAACCCCAAAGCCAACAGGGAGAAGATGACGCAG  480
     P  V  L  L  T  E  A  P  L  N  P  K  A  N  R  E  K  M  T  Q
481  ATCATGTTCGAGACCTTCAACACCCCGGCCATGTACGTCGCCATCCAAGCCGTGCTGTCT  540
     I  M  F  E  T  F  N  T  P  A  M  Y  V  A  I  Q  A  V  L  S
541  CTGTACGCCTCTGGCCGCACCACCGGTATCGTCTTGGACTCTGGCGACGGCGTCTCCCAC  600
     L  Y  A  S  G  R  T  T  G  I  V  L  D  S  G  D  G  V  S  H
601  ACCGTTCCCATCTACGAGGGTTACGCCCTTCCTCACGCCATCTTGCGTCTGGATTTGGCC  660
     T  V  P  I  Y  E  G  Y  A  L  P  H  A  I  L  R  L  D  L  A
661  GGACGTGACTTGACCGACTATCTCATGAAGATCTTGACCGAGAGAGGCTACTCGTTCACC  720
     G  R  D  L  T  D  Y  L  M  K  I  L  T  E  R  G  Y  S  F  T
721  ACCACCGCCGAGCGTGAGATCGTTCGTGACATCAAAGAGAAGCTCTGCTACGTGGCGTTG  780
     T  T  A  E  R  E  I  V  R  D  I  K  E  K  L  C  Y  V  A  L
781  GACTTCGAGCAGGAGATGGCGACCGCCGCCTCCTCTTCCTCTTTGGAGAAGTCCTACGAA  840
     D  F  E  Q  E  M  A  T  A  A  S  S  S  S  L  E  K  S  Y  E
841  TTGCCCGACGGTCAAGTCATCACCATCGGCAACGAGCGATTCCGCTGCCCCGAGGCCCTC  900
     L  P  D  G  Q  V  I  T  I  G  N  E  R  F  R  C  P  E  A  L
901  TTCCAGCCGTCCTTCCTCGGCATGGAATCTTGCGGCATCCACGAGACTACCTACAACTCC  960
     F  Q  P  S  F  L  G  M  E  S  C  G  I  H  E  T  T  Y  N  S
961  ATCATGAAGTGCGACGTCGACATCCGTAAGGATCTTTACGCCAACACCGTCTTGTCCGGT  1020
     I  M  K  C  D  V  D  I  R  K  D  L  Y  A  N  T  V  L  S  G
1021 GGCACCACCATGTACCCCGGCATCGCCGACCGCATGCAGAAAGAGATCACAGCCCTGGCT  1080
     G  T  T  M  Y  P  G  I  A  D  R  M  Q  K  E  I  T  A  L  A
1081 CCCTCCACCATGAAGATCAAGATCATCGCTCCCCCGGAGAGGAAGTACTCCGTCTGGATC  1140
     P  S  T  M  K  I  K  I  I  A  P  P  E  R  K  Y  S  V  W  I
1141 GGAGGCTCCATCCTGGCCTCCCTGTCCACCTTCCAACAGATGTGGATCTCCAAGCAAGAG  1200
     G  G  S  I  L  A  S  L  S  T  F  Q  Q  M  W  I  S  K  Q  E
1201 TACGACGAGTCTGGTCCGTCGATCGTCCACAGGAAATGCTTCTAAATCTAACAAAACTAA  1260
     Y  D  E  S  G  P  S  I  V  H  R  K  C  F  *
1261 TGATTTTGACTTTAAAACCGCCGCGCGTCCCGACATTTTTTTTTCTCTCTTTGAATTAGA  1320
1321 AATTAGGAAATCTCGTTCCCTTTTCTTTTTTATTTATTGCTAGTCGGTTTTTTTTATTCT  1380
1381 TTCGGAAAGATGTTTTTTTTTTCCACTTCACATCCTTGTTATCGCGTTTAAAAACAAAAC  1440
1441 AAAAATTATATGATATTGTTCGAGTTTATTTGACCTTGTACGCTTGGCAAAGGAATTAAA  1500
1501 ATTTGAAACATACTTCGAAACATTTCCATCGCCAATTAATAACTCTATAATAAAGCCAAA  1560
1561 TTGATTTTTACAATTTGCCGCCCTACCAAAAATTTGCGTTACTGTCTTTTTGGAGCGATT  1620
1621 CTTGTACTATGCATGTGAATATAAGTTCTTTTTAGCTTCAAAAAAAAAAAAAAAAA  1677
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Fig. 1. Nucleotide and predicted amino acid sequences of the cDNA clone for the *PgAct1* gene. The sequence of the cDNA encompasses 1,677 bp including 18 adenylyl residues at the 3' end. The ATG at the position 115-117 represents the putative start codon of the *PgAct1*-encoded protein. The asterisk indicates the termination codon. The nucleotide sequence will appear in the DDBJ, EMBL and GenBank Nucleotide Sequence Databases with the accession number (D45164).

or sense probe for at least 16 hr at 42°C. After hybridization, the specimens were washed and treated with RNase A and then washed again extensively with PBT. The samples were then incubated for 1 hr with 1:2000 Boehringer Mannheim alkaline-phosphatase-conjugated anti-DIG and treated for the development of color as indicated in the protocol from Boehringer. After dehydration, some of the specimens were cleared by placing them in a 1:2 mixture (v/v) of benzyl alcohol and benzyl benzoate.

Sectioned specimens: These specimens were embedded in polyester wax and sectioned at 10 μm. The *in situ* hybridization of sectioned specimens was carried out as in the case of whole-mount specimens.

RESULTS AND DISCUSSION

Isolation and characterization of a cDNA clone for *Paraspadella gotoi* actin genes

We first made first-stranded cDNAs against *P. gotoi* adult RNAs. Then, using oligonucleotide primers corresponding to the shared amino-acid sequences of actins, we amplified target fragments from them by the RT-PCR reaction. We obtained 12 amplified fragments which were about 1.1-kb long and corresponded to the region from the amino acid position 10 to 375 of actins. Sequencing the fragments after subcloning them into pBluescript II SK(+) revealed that these 12 clones were subdivided into three types (Types-1, -2, and -3); 6 clones were of Type-1, 3 were of Type-2, and 3 were of Type-3.

With labeled clones, we screened 2.4×10^5 pfu of the *P. gotoi* adult cDNA library and obtained 42 positive clones. The partial sequencing of these clones revealed that 10 contained sequences identical to Type-1, 5 to Type-2, and 6 to Type-3. In addition to these three types, there were seven different types of 12 clones, which were not analyzed further. Of the clones belonging to each of the three types, the longest ones were entirely sequenced. Each of the longest clones contained a single open reading frame (ORF) that predicted actins.

```
1     GTTCGACGCATCTCTTGATCCGACAGCGAATCTCGCCTCTTTCTTTTTTGTCGTCGTT   58
59    TGAAGACAACAACAACAACAACCAGCCAAGATGTGCGACGATGAGGAGGTAGCGGCGTTG  118
                          M  C  D  D  E  E  V  A  A  L
119   GTCGTCGACAATGGATCGGGAATGTGCAAGGCCGGGTTCGCCGGCGACGACGCCCCCGC  178
      V  V  D  N  G  S  G  M  C  K  A  G  F  A  G  D  D  A  P  R
179   GCCGTCTTCCCCTCCATCGTCGGACGCCCTCGTCATGTGGGCGTCATGGTGGGCATGGGG  238
      A  V  F  P  S  I  V  G  R  P  R  H  V  G  V  M  V  G  M  G
239   CAGAAGGACGCCTATGTGGGCGACGAGGCTCAGTCCAAGAGAGGTATCCTCTCTCTGAAA  298
      Q  K  D  A  Y  V  G  D  E  A  Q  S  K  R  G  I  L  S  L  K
299   TACCCCATTGAGCACGGCATCGTCACCAACTGGGACGACATGGAGAAGATCTGGCATCAC  358
      Y  P  I  E  H  G  I  V  T  N  W  D  D  M  E  K  I  W  H  H
359   ACCTTCTACAACGAGTTGAGAGTCTCTCCCGAGGAGCACCCCGCTCTCCACTCGGAGGCG  418
      T  F  Y  N  E  L  R  V  S  P  E  E  H  P  A  L  H  S  E  A
419   CCCCTCAACCCTAAGGCCAACAGGGAGAAGATGACCCAAATCTGCTTCGAGGCTTTCAGC  478
      P  L  N  P  K  A  N  R  E  K  M  T  Q  I  C  F  E  A  F  S
479   GCCCCCGCGATGTACGTAGCCATTCAGGCTGTGCTGTCGCTGTACGCTTCCGGCCGCACG  538
      A  P  A  M  Y  V  A  I  Q  A  V  L  S  L  Y  A  S  G  R  T
539   ACCGGCATCGTGCTGGACACCGGCGACGGTGTCGCTCACGCCGTCCCCATCTACGAGGGT  598
      T  G  I  V  L  D  T  G  D  G  V  A  H  A  V  P  I  Y  E  G
599   TACGCCCTCCCTCACGCCATCCTCAGGCTCGACCTCGCCGGGAGGGATCTCACCGACTAC  658
      Y  A  L  P  H  A  I  L  R  L  D  L  A  G  R  D  L  T  D  Y
659   CTCATGAAGATCCTCACCGAGAGGGGATATTCCTTCGTGACGACGGCCGAGCGCGAGATT  718
      L  M  K  I  L  T  E  R  G  Y  S  F  V  T  T  A  E  R  E  I
719   GTCCGCGACATCAAGGAGAAGCTCTGCTACGTGGCGCTCGACTTCGAAAACGAGATGAAC  778
      V  R  D  I  K  E  K  L  C  Y  V  A  L  D  F  E  N  E  M  N
779   ATCGCCAAATCGTCTTCGTCGCTCGAGAAGTCCTACGAGTTGCCCGACGGTCAGGTCATC  838
      I  A  K  S  S  S  S  L  E  K  S  Y  E  L  P  D  G  Q  V  I
839   ACCGTCGGCAACGAGCGATTCCGCTGCCCAGAGGCCATCTTCCAGCCTTCCTTCTTGGGA  898
      T  V  G  N  E  R  F  R  C  P  E  A  I  F  Q  P  S  F  L  G
899   ATGGAGATCGTCGGTGTTCACGAGGGATGCTTCAACAGCATCATGAAGTGCGACATCGAC  958
      M  E  I  V  G  V  H  E  G  C  F  N  S  I  M  K  C  D  I  D
959   ATCCGTAAGGATCTCTACGCCAACACCGTCCTGTCCGGAGGCACCACCATGTACCCCGGT 1018
      I  R  K  D  L  Y  A  N  T  V  L  S  G  G  T  T  M  Y  P  G
1019  ATCGCCGACCGCATGCAGAAGGAGATCACCGCCCTGGCTCCCTCCACGATGAAGATCAAG 1078
      I  A  D  R  M  Q  K  E  I  T  A  L  A  P  S  T  M  K  I  K
1079  ATCATCGCTCCCCCGGAGAGGAAGTATTCCGTCTGGATCGGCGGTTCCATCCTGGCCTCC 1138
      I  I  A  P  P  E  R  K  Y  S  V  W  I  G  G  S  I  L  A  S
1139  CTGTCCACCTTCCAGGAGATGTGGATCTCCAAGCAGGAGTACGACGAGGCCGGCCCGGGC 1198
      L  S  T  F  Q  E  M  W  I  S  K  Q  E  Y  D  E  A  G  P  G
1199  ATCGTCCACAGGAAGTGCTTCTAGACGAAACGCGGGAGACTCCCACCCAGCGTCGTAGTT 1258
      I  V  H  R  K  C  F  *
1259  TGTAGCCGAGATTTTTTTAAATTTTTTTATATTTGTTTAATTTTTGATTTTTTTTTTTTT 1318
1319  CGTATAAAGTTTTACTGTATCGAAAAAAAAACCAAACATGTCTTTAAAAAAACATCGCGA 1378
1379  AACCTTGTTCTGATTAAAATACGTTGACACAAACCTACAATTGCATGTTCGTTGATGACA 1438
1439  TTCCTGTACTGGTGCATACAAATCGAAATGGTTCTGATCCCTTCATCTCATCGTCCGATT 1498
1499  AAATTTATTTAAGTTCAAAAAAAAAAAAAAAAAAA 1534
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Fig. 2. Nucleotide and predicted amino acid sequences of the cDNA clone for the *PgAct2* gene. The sequence of the cDNA encompasses 1,534 bp including 20 adenylyl residues at the 3' end. The ATG at the position 89-91 represents the putative start codon of the *PgAct2*-encoded protein. The asterisk indicates the termination codon. The nucleotide sequence will appear in the DDBJ, EMBL and GenBank Nucleotide Sequence Databases with the accession number (D45165).

```
1     CGCTCCTCCAGCGTATTCCCCGAATCTTTCGACAGACGCGAAGTAAACCTCCAGGATG   58
                                                            M
59    TGCGACGACGAGGAATCCGCGGCGTTGGTCATCGATAATGGATCCGGAATGGTCAAGGCC  118
      C  D  D  E  E  S  A  A  L  V  I  D  N  G  S  G  M  V  K  A
119   GGGTTCGCCGGCGACGACGCCCCCGCGCCGTCTTCCCCTCCGTCGTCGGACGCCGCGT  178
      G  F  A  G  D  D  A  P  R  A  V  F  P  S  V  V  G  R  P  R
179   CACGTCAGCGTCATGGTGGGCATGGGGAACAAAGACGCCTACGTCGGTGACGAGGCTCAG  238
      H  V  S  V  M  V  G  M  G  N  K  D  A  Y  V  G  D  E  A  Q
239   TCCAAGAGAGGTATCCTCTCCCTGAAGTACCCCATCGAGCACGGCATCGTCACCAACTGG  298
      S  K  R  G  I  L  S  L  K  Y  P  I  E  H  G  I  V  T  N  W
299   GACGATATGGAGAAAGTGTGGCATCACACCTTCAACAACGAGCTGCGCATCTCCCCCGAG  358
      D  D  M  E  K  V  W  H  H  T  F  N  N  E  L  R  I  S  P  E
359   GAATCGCCCCATCTCCACTCCGAGGCGCCCCTCAACCCAAGAGCAACAGGGAGAAGCTC  418
      E  S  P  H  L  H  S  E  A  P  L  N  P  K  S  N  R  E  K  L
419   GTCCAGATTGTCTTCGAGACCTTCAACGCCCCCGCCACGTACGTCTGTATCCAGGCCGTA  478
      V  Q  I  V  F  E  T  F  N  A  P  A  T  Y  V  C  I  Q  A  V
479   CTCTCCCTGTACGCCTCTGGCCGTACGACCGGCATGGTGTTGGACATCGGAGACGGTGTC  538
      L  S  L  Y  A  S  G  R  T  T  G  M  V  L  D  I  G  D  G  V
539   TCCCACGGCGTCCCCATCTACGAGGGTTACGCTCTCCCTCACGCCATCTTGCGTCTCGAT  598
      S  H  G  V  P  I  Y  E  G  Y  A  L  P  H  A  I  L  R  L  D
599   TTGGCCGGACGCGACTTGACCGACTATCTCATGAAGATCATGTCGGAGCGTGGCTACGCC  658
      L  A  G  R  D  L  T  D  Y  L  M  K  I  M  S  E  R  G  Y  A
659   ATGGTCACCACCGCCGAGCGCGAGATCGTCCGGGACATCAAGGAGAAGCTTTGCTACGTG  718
      M  V  T  T  A  E  R  E  I  V  R  D  I  K  E  K  L  C  Y  V
719   GCGCTGGACTTCGAGCAGGAAATGGCGACCGCCGCCTCCTCCTCCTCCATTGACAAGTCC  778
      A  L  D  F  E  Q  E  M  A  T  A  A  S  S  S  S  I  D  K  S
779   TACGAGTTGCCCGACGGACAGATCGTGACCGTCGGCAACGAGCGTTTCCGTTGCCCCGAA  838
      Y  E  L  P  D  G  Q  I  V  T  V  G  N  E  R  F  R  C  P  E
839   TCCATGTTCCGCCCCAACTTCCTCGGCATGGAAGTCGTCGGAATTCACGAGGGATGCTTC  898
      S  M  F  R  P  N  F  L  G  M  E  V  V  G  I  H  E  G  C  F
899   AACGGAATCATGAAGTGCGACATCGACATCCGCAAAGATCTCTACGCCAACACCGTCTTG  958
      N  G  I  M  K  C  D  I  D  I  R  K  D  L  Y  A  N  T  V  L
959   TCCGGTGGCACCACCATGTACCCCGGTATCGCTGACCGCATGCAGAAAGAGATCACCGCC 1018
      S  G  G  T  T  M  Y  P  G  I  A  D  R  M  Q  K  E  I  T  A
1019  CTGGCTCCCTCCACCATGAAGATCAAGATCATCGCTCCCCCGGAGAGGAAGTACTCCGTC 1078
      L  A  P  S  T  M  K  I  K  I  I  A  P  P  E  R  K  Y  S  V
1079  TGGATCGGCGGTTCCATCCTGGCCTCCCTGTCCACCTTCCAGGAGATGTGGATCTCCAAG 1138
      W  I  G  G  S  I  L  A  S  L  S  T  F  Q  E  M  W  I  S  K
1139  CAAGAGTACGACGAGGCTGGACCCGGCATCGTGCACAGGAAATGCTTCTAAACGAATCCA 1198
      Q  E  Y  D  E  A  G  P  G  I  V  H  R  K  C  F  *
1199  CCGATTAACAACACCCACTAAACGCGCACACACACACAAAAATAAAATTATTGTATTTAT 1258
1259  TTTTGCTATTCTTTAATTTTTTTTCACATTTTTGTTGATCTCTCGCGTTGGGAATGAGTT 1318
1319  TCAAGATCTTTGCCGTCGAACCGAGGAAAAAAAATCTGTCATCGAAACGAAGAGCGAATC 1378
1379  TTTCACGGTTCTGCGAAACAAAGAACTTGAAATTTATTTTTCGAAATTTGTTCGATT 1438
1439  CGATTTTTAAAAAAATATGTGGTCGGATTTTTCGGAATTAAAAGTTTCCCAACGAAAAAA 1498
1499  AAAAAAAAAAA 1510
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Fig. 3. Nucleotide and predicted amino acid sequences of the cDNA clone for the *PgAct3* gene. The sequence of the cDNA encompasses 1,510 bp including 18 adenylyl residues at the 3' end. The ATG at the position 56-58 represents the putative start codon of the *PgAct3*-encoded protein. The asterisk indicates the termination codon. The nucleotide sequence will appear in the DDBJ, EMBL and GenBank Nucleotide Sequence Databases with the accession number (D45166).

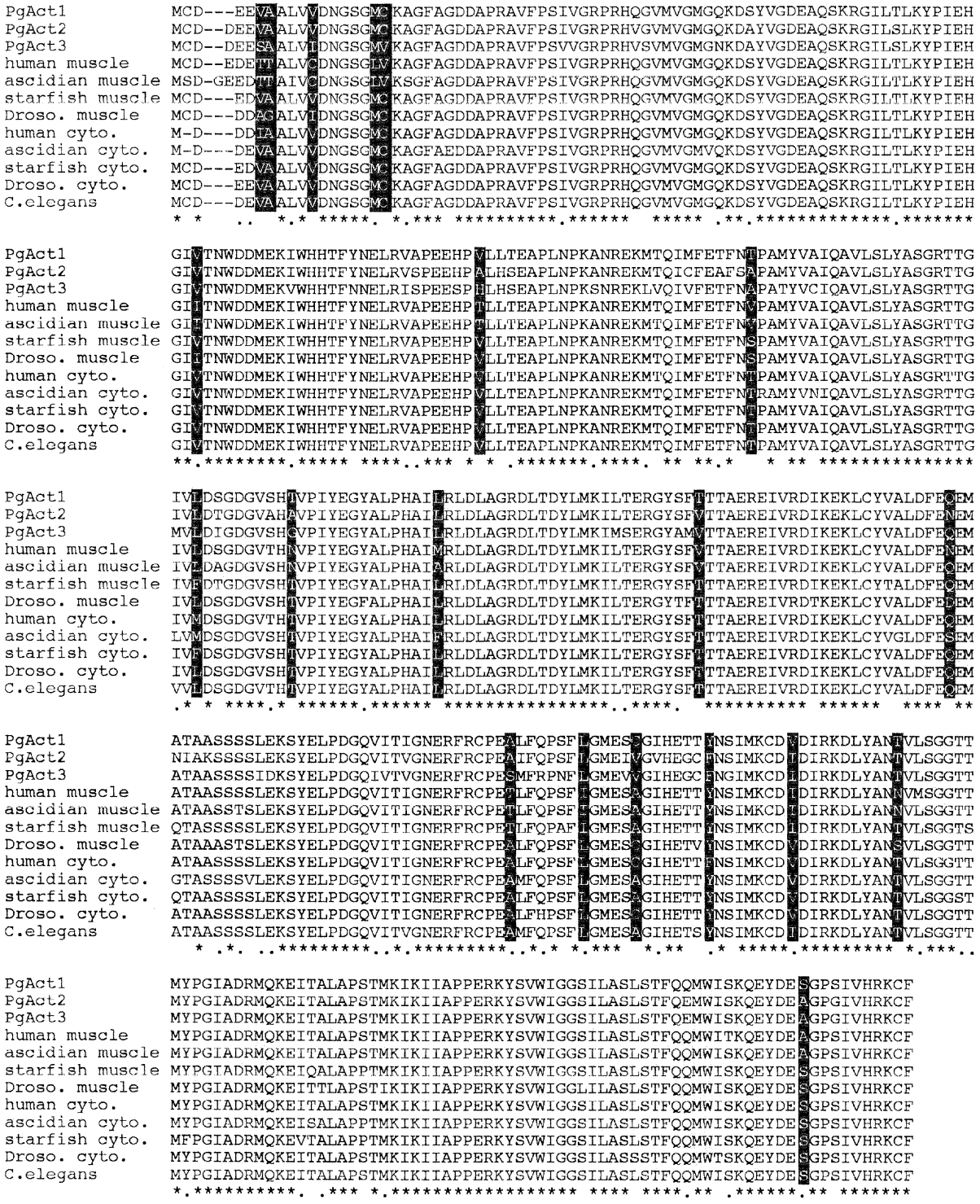

Fig. 4. Comparison of the amino acid sequences of PgAct1, PgAct2, and PgAct3 with actins of various metazoans. The amino acids are indicated with one-letter codes. Dashes indicate gaps introduced in the sequence to optimize the alignment. The amino acids at diagnostic positions indicating vertebrate actin types (cytoplasmic or muscle) are boxed (Vandekerckhove and Weber, 1978, 1984). The asterisks indicate the identity of amino acids, and the dots indicate high similarity. The sources, accession numbers, and references for the actin sequences are human α-skeletal muscle actin, M20543 (Taylor *et al.*, 1988); ascidian larval muscle actin, D10887 (Kusakabe *et al.*, 1992); starfish muscle actin, M26500 (Kowbel and Smith, 1989); *Drosophila* muscle actin, M18830 (Sanchez *et al.*, 1983); human β-cytoplasmic actin, M10277 (Nakajima-Iijima *et al.*, 1985); ascidian cytoplasmic actin, X61042 (Kovilur *et al.*, 1993); starfish cytoplasmic actin, M26501 (Kowbel and Smith, 1989); *Drosophila* cytoplasmic actin, K00667 (Fyrberg *et al.*, 1981) and *C. elegans* actin, X16796 (Krause *et al.*, 1989).

We therefore named the corresponding genes *PgAct1* (*Paraspadella gotoi actin* gene 1) to Type-1, *PgAct2* to Type-2, and *PgAct3* to Type-3.

Figure 1 shows the nucleotide and deduced amino acid sequences of the cDNA clone for the *PgAct1* gene, which consisted of 1,677 nucleotides. The *PgAct1* cDNA contained a single ORF of 1,128 bp, which predicted a polypeptide of 376 amino acids. The calculated molecular mass of the predicted protein was 41.8 kDa.

The nucleotide and deduced amino acid sequences of the cDNA clone for the *PgAct2* and *PgAct3* genes are shown in Figs. 2 and 3. The *PgAct2* cDNA was 1,534 bp long and contained a single ORF of 1,128 bp, which predicted a polypeptide of 377 amino acids and had a calculated molecular mass of 42.3 kDa, while the *PgAct3* cDNA was 1,510 bp long and contained a single ORF of 1,128 bp, which predicted a polypeptide of 377 amino acids and 42.3 kDa.

Among these three actins, the amino acid identities were 91.5% between PgAct1 and PgAct2, 86.2% between PgAct1 and PgAct3, and 88.9% between PgAct2 and PgAct3, although the identity of the nucleotide sequences of 3' untranslated region (UTR) was less than 50%.

Figure 4 shows a comparison of the amino acid sequences of PgAct1, PgAct2 and PgAct3 with actins of various metazoans. The comparison clearly indicated that the *PgAct1*, *PgAct2*, and *PgAct3* genes encode different types of actin. As shown in Fig. 4, the mammalian α-striated muscle actin is distinguishable from the β-cytoplasmic actin by a comparison of amino acid residues at the diagnostic positions (Vandekerckhove and Weber, 1978, 1984). Figure 4 suggests that PgAct1 is of a type of cytoplasmic actin and that PgAct2 and PgAct3 are considerably divergent types of actin. Our molecular phylogenetic analysis comparing the amino acid sequences also suggested that PgAct2 and PgAct3 are closely related, while PgAct1 is distinct from them, that PgAct1 is a type of cytoplasmic actin, and that PgAct2 and PgAct3 are divergent types of actin (Yasuda *et al.*, unpublished data).

These results suggest that the genome of *P. gotoi* contains a family of multiple actin genes and that among them, *PgAct1*, *PgAct2*, and *PgAct3* are major actin genes judging from the numbers of cDNA clones isolated. Because 6 of the 12 amplified fragments and 10 of the 42 positive cDNA clones corresponded to *PgAct1*, this gene may represent the most dominant actin gene of *P. gotoi*. *PgAct2* and *PgAct3* genes are also actively expressed in this animal, judging from the numbers of cDNA clones obtained.

Expression of *Paraspadella gotoi* actin genes

The expression of the arrow worm actin genes was examined by *in situ* hybridization with sectioned specimens of adults and whole-mount specimens of juveniles. To help clarify the gene expression, the anatomy of *P. gotoi* is diagrammatically shown in Fig. 5a. Their trimeric body is comprised of a head, trunk and postanal tail divided from one another by transverse septa. The head cavity is reduced by the complex cephalic musculature. The body musculature consists of four quadrants of well-developed dorsolateral and ventrolateral longitudinal bands. The digestive tract runs through the central part of the trunk region and is completed by the anus at the ventral surface of the trunk-tail junction. A major component of the central nervous system is a large ventral ganglion located in the trunk epidermis. Arrow worms are hermaphroditic; the wide coelom of the trunk region is occupied with oogenic cells, while that of the tail region occupied by spermatogenic cells. Adults were examined in cross-sections of the head, trunk, and tail regions.

The *PgAct1* gene. The *in situ* hybridization of adult specimens demonstrated intense signals in oogenic cells of the trunk region (Fig. 5c). Small oocytes showed stronger signals, whereas large mature oocytes showed weak signals (Fig. 5c). In addition, sections of the tail region revealed signals in the spermatogenic cells or spermatocytes (Fig. 5d). Therefore, the *PgAct1* gene is expressed in germ cells during the early phase of their formation.

Hybridization signals were also evident in the cytoplasm of the neuronal cells of the ventral ganglion (Fig. 5c) as well as in cells of the digestive tract including the pharynx in the head region (Fig. 5b) and intestine in the trunk region (Fig. 5c). However, signals were not so evident in longitudinal muscle cells (Fig. 5c, d).

The *in situ* hybridization of the whole-mount specimens of newly-hatched juveniles showed intense signals on the whole body except for the tail region (Fig. 5e). Strong signals were seen in the head region (Fig. 5e).

These results suggest that the *PgAct1* gene is expressed both maternally and zygotically. The gene is expressed in a variety of tissues, supporting the notion that PgAct1 is a type of cytoplasmic actin.

The *PgAct2* gene. Cross-sections of the head region of the adults demonstrated that the *PgAct2* gene was actively expressed in well-developed muscle of the gnatha region (Fig. 6a). Hybridization signals were also evident on the epithelium lining the coelom near the ventral ganglion (Fig. 6b) and in cells of the sperm duct of the trunk region (Fig. 6b). However, signals were not detected in the pharynx (Fig. 6a, b) or germ cells (Fig. 6b, c).

The *in situ* hybridization of whole-mount specimens of newly-hatched juveniles showed intense signals in the head region, in particular in the gnathic region (Fig. 6d). Lateral cells in the trunk region also showed signals (Fig. 6d).

These results suggest that the *PgAct2* gene expression is zygotic, and primarily found in muscle cells.

The *PgAct3* gene. In contrast to the *PgAct2* gene, the *in situ* hybridization of sectioned specimens of adult tissues demonstrated intense signals in oogenic cells of the trunk region (Fig. 6f). The small oocytes showed very strong signals (Fig. 6f). Signals were also evident in longitudinal muscle cells of the trunk and tail regions (Fig. 6f, g). In the tail region, signals were evident in the mesentery (Fig. 6g). The *in situ* hy-

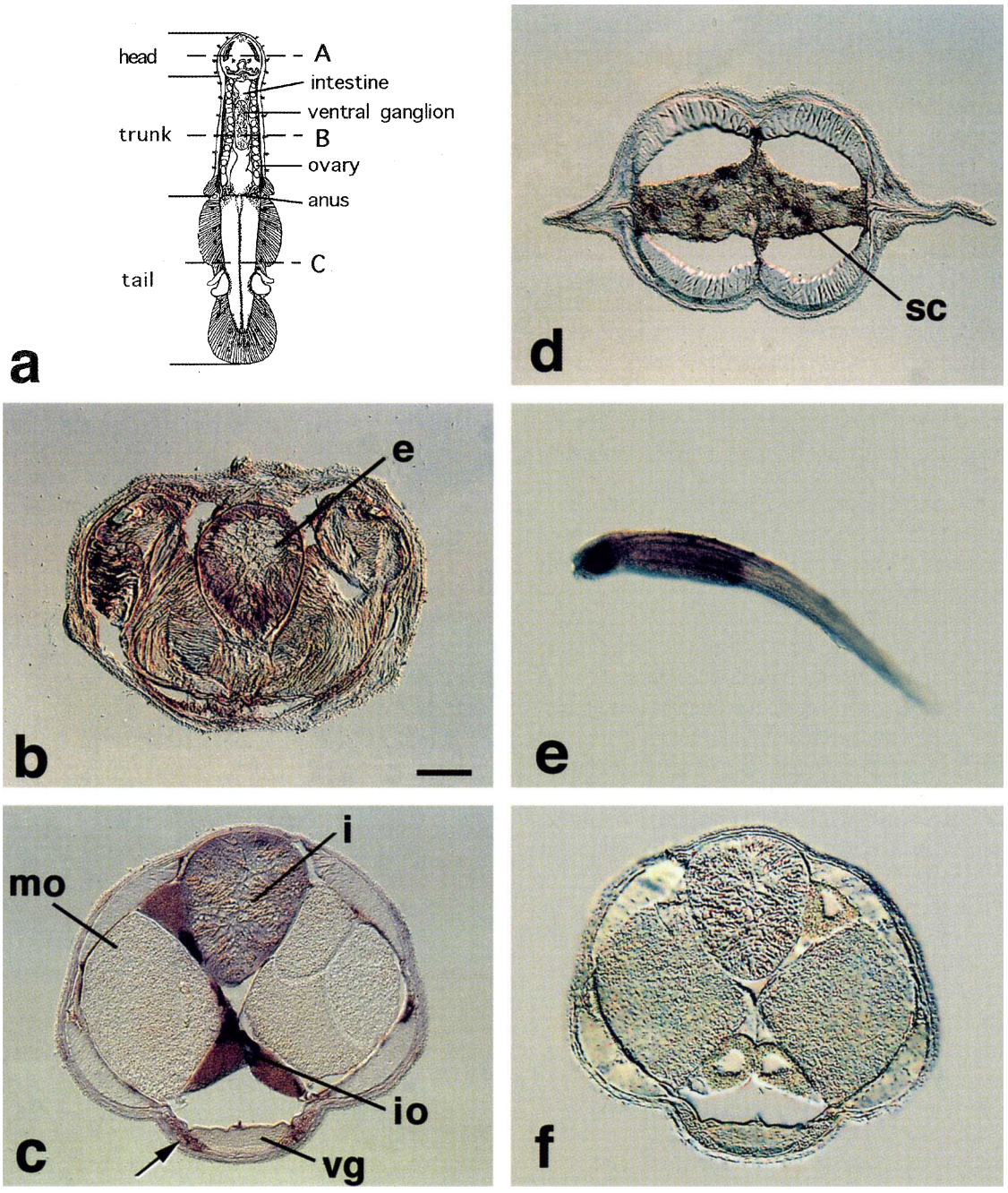

Fig. 5. Expression of the *PgAct1* gene. (**a**) Diagrammatic drawing of *P. gotoi* showing the anatomy and the sectioned lines; A, a section at the head region; B, a section at the trunk region; and C, a section at the tail region. (**b**) A cross-section of the head region of an adult, showing a weak hybridization signal in the esophagous (e). Scale bars = 100 μm for all panels. (**c**) A cross-section of the trunk region of an adult, showing a strong signal in the immature oocytes (io) but not in the mature oocytes (mo). Signals were also evident in the intestine (i) and neuronal cells (arrow) in the ventral ganglion (vg). (**d**) A cross-section of the tail region of an adult, showing a hybridization signal in some of the spermatocytes (sc). (**e**) A whole-mount specimen of a juvenile, showing signals widely distributed in the head and trunk regions. (**f**) A control cross-section of the trunk region of an adult (corresponding to **c**) hybridized with a sense probe, showing no signals.

Fig. 6. (**a-d**) Expression of the *PgAct2* gene. (**a**) A cross-section of the head region of an adult, showing a strong hybridization signal in the muscle (m) of the gnatha region. Scale bars = 100 μm for all panels. (**b**) A cross-section of the trunk region of an adult, showing signals in the seminiferous duct region (upper arrow) and in the epithelium lining the coelom of the ventral ganglion region (lower arrow). (**c**) A cross-section of the tail region of an adult, showing no hybridization signal in this region. (**d**) A whole-mount specimen of a juvenile, showing distinct signals in the head muscle (left arrow) and trunk muscle (right arrow). (**e-h**) Expression of the *PgAct3* gene. (**e**) A cross-section of the head region of an adult, showing no hybridization signal in this region. (**f**) A cross-section of the trunk region of an adult, showing a strong signal in the immature oocytes (io) and muscle cells (arrow). (**g**) A cross-section of the tail region of an adult, showing a distinct signal in the muscle (m). Signals are evident in muscle cells (arrow). (**h**) A whole-mount specimen of a juvenile, showing strong signals over the entire body.

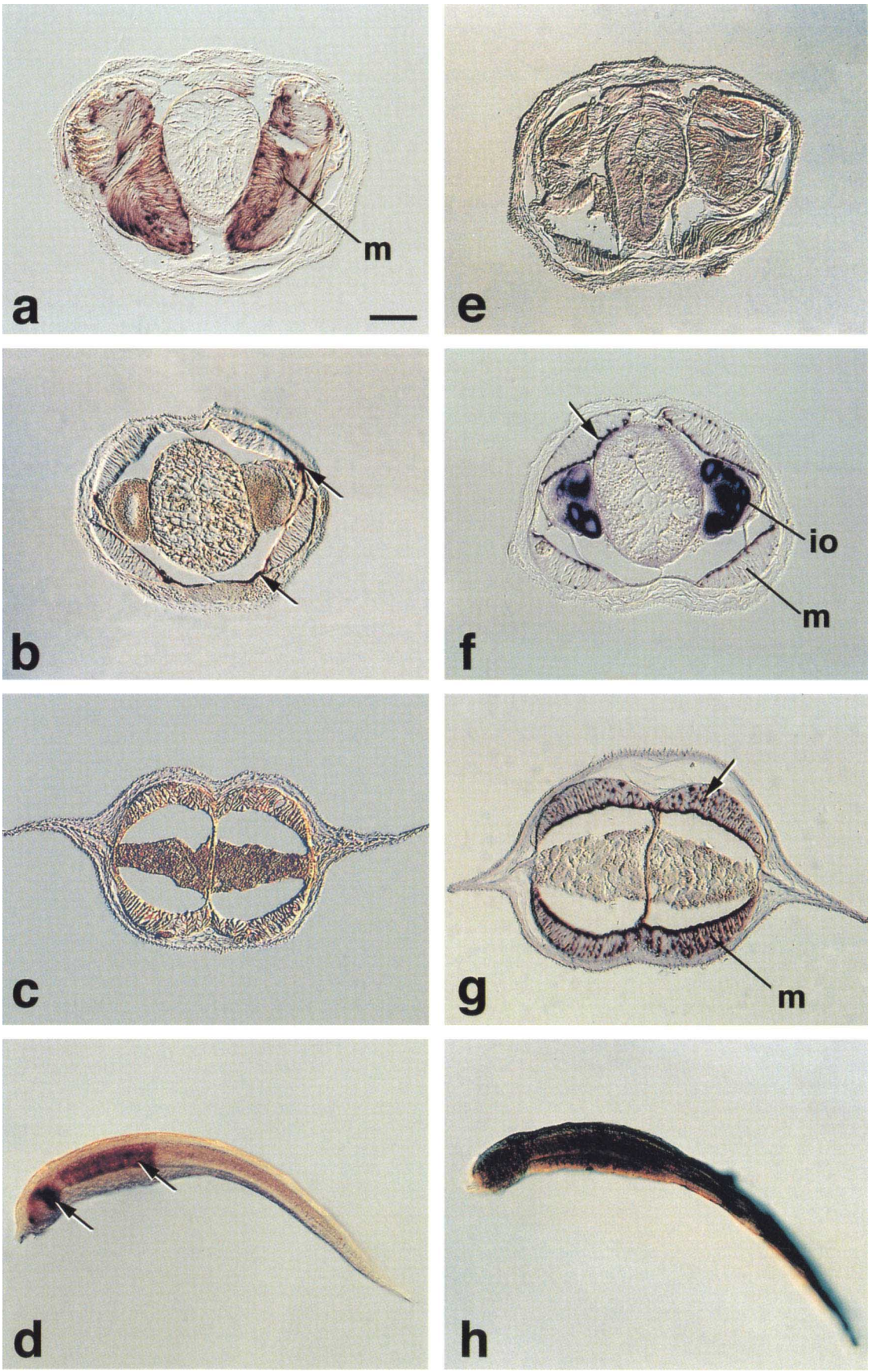

Table 1. Spatial expression of the three actin genes *PgAct1*, *PgAct2*, and *PgAct3* of the arrow worm *Paraspadella gotoi*

	Adult						Juvenile		
	head muscle	trunk muscle	oocytes	spermduct	neuronal cells	spermato-cytes	head region	trunk region	tail region
PgAct1	–	–	+++	–	++	+	++	+	–
PgAct2	++	–	–	+	–	–	+++	++	–
PgAct3	–	++	+++	–	–	+	++	+	++

–, not expressed; +, weakly expressed; ++, moderately expressed; +++, strongly expressed

bridization of whole-mount specimens of newly-hatched juveniles showed intense signals on the whole body (Fig. 6h).

These results suggest that the *PgAct2* gene is primarily expressed in the longitudinal muscle as well as oocytes.

The regions with expressions of *PgAct1*, *PgAct2* or *PgAct3* are summarized and compared in Table 1. As is evident in the table, the patterns of spatial expression of the three actin genes are not identical but rather are specific for each gene. It is highly likely that each of these genes has its own function.

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