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Original Article

HrWnt-5: a maternally expressed ascidian *Wnt* gene with posterior localization in early embryos

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ABSTRACT Ascidians show a highly determinate mode of development. In particular, components of the posterior-vegetal cytoplasm of fertilized eggs are responsible for the establishment of the embryonic axis. Recent studies have, however, also revealed significant roles of cell-cell interactions during embryogenesis. Proteins encoded by the *Wnt* family of genes act as signals and have been shown to play important roles in a wide range of developmental processes. Here we have isolated and characterized an ascidian *Wnt* gene, *HrWnt-5,* from *Halocynthia roretzi. HrWnt-5* mRNA is present in the vegetal cortex in unfertilized eggs. After fertilization, *HrWnt-5* mRNA moves to the equatorial region to form a crescent-like structure, after which the mRNA is concentrated in the posteriormost region of the embryo. This early pattern of *HrWnt-5* mRNA localization coincides with another posterior-vegetally localized mRNA, *pem,* isolated from *Ciona savignyi.* In the gastrula, the zygotic *HrWnt-5* mRNA is found in a variety of blastomeres, suggesting multiple roles of the gene.

KEY WORDS: *ascidian, maternal mRNA, Wnt5, posterior*

Introduction

The development of an organism requires the establishment of a series of positional cues, with which the proper formation and correct positioning of many structures are achieved. The cues are, unless derived externally, ultimately originated from maternal information confined to a particular region of the egg cytoplasm (Davidson, 1986). Numerous maternal genetic programs and factors responsible for the body plan of *Drosophila* (reviewed by St. Johnston and Nüsslein-Volhard, 1992) and for early cell specification of nematode (reviewed by Bowerman, 1995) have been reported. There is, however, a gap of knowledge regarding the relationships between maternal information and the establishment of positional cues, particularly in chordate embryos.

Ascidians are one of the most basal chordate. Ascidian embryos are a useful experimental system with which the genetic circuitry required for cell specification and morphogenesis has been explored (Satoh, 1994). The ascidian egg has been regarded as a typical 'mosaic' egg which shows a highly determinate mode of development (Nishida, 1992,1993,1994a) since the first blastomere destruction experiment was described in the history of embryology (Chabry, 1887), whereas cell-cell signaling also plays crucial roles during early embryogenesis (Nishida, 1991; Nakatani and Nishida, 1994; Miya *et al.*, 1996). Nishida (1994b) showed that the posterior-vegetal cytoplasm in fertilized eggs has functions in processes including muscle formation, the suppression of anterior fate, the generation of a posterior cleavage pattern and the morphogenesis of tail formation, suggesting that the establishment of anteroposterior asymmetry depends on prelocalized egg cytoplasmic factors. However, the identity and action of the factors involved in the anteroposterior axis formation in the ascidian embryo remains unknown. Yoshida *et al.* (1996) reported that a maternally derived *posterior end mark (pem)* mRNA is localized in the posterior-vegetal cytoplasm in *Ciona* embryos, and that the overexpression of synthetic transcripts in entire fertilized eggs affected the anterior and dorsal structures of the larva but did not disturb the other above-mentioned processes. A novel subcellular structure called a centrosome-attracting body was recently found in the posterior-vegetal cytoplasm in the *Halocynthia* embryo (Hibino *et al.*, 1998). This apparatus seems to play a direct role in the unequal cell division which results in the posterior cleavage pattern (Nishikata *et al.*, personal communication). Thus, it is likely that different factors are responsible for the distinct properties of the posterior-vegetal cytoplasm.

To obtain molecular probes with which the mechanisms underlying these processes can be revealed, we have started a mass screening of cDNAs in a fertilized egg of *Halocynthia roretzi* for prelocalized messages.

In this study, we report the isolation and characterization of a cDNA that encodes an ascidian homolog *(HrWnt-5)* of vertebrate

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   1  CTAACGTGTAAAAAGGTTGGCCTTGTTTGTGCTGAAGACTGAACTGAACATTATTGACAGTTGAGTGTTGTTAGTCGCAAACGTGGTTTG  90
                *                   *         *                    *
  91  GAATCCCCGAAGACGACAATACATAATAAATACATCAGAGAAATCGTACAGAAATGGTCGGAATGACAAGAATACAATCTGCAGAGCCCG 180
                                  * *                      M  V  G  M  T  R  I  Q  S  A  E  P   12
 181  TCTGGATTTTATTTGTTTTAACTCTCTATTCGTCAGTTTTGATGCAAGTCAAGCCTCAGTTATGGTCTGTTGGCATCGAAAGAAAGAAAC 270
  13  V  W  I  L  F  V  L  T  L  Y  S  S  V  L  M  Q  V  K  P  Q  L  W  S  V  G  I  E  R  K  K   42
 271  TCTTTGGAGAAACGAACACGTCCGTTCACTGCGATGAAATCCGCGGACTTTCAAGAAACCAACGGTCGCTATGTCGTACCTACAACGACC 360
  43  L  F  G  E  T  N  T  S  V  H  C  D  E  I  R  G  L  S  R  N  Q  R  S  L  C  R  T  Y  N  D   72
 361  ATATGTATTATGTTGAAAGTGGATCCAAGCAAGGGGTGGAGGAATGCCAGTGGCAGTTTCGAGGGCAACGATGGAACTGTTCGCTCGCTT 450
  73  H  M  Y  Y  V  E  S  G  S  K  Q  G  V  E  E  C  Q  W  Q  F  R  G  Q  R  W  N  C  S  L  A  102
 451  CCAACGCTTCGCCTGATAAAATTATTGCCGTCGGAAGTAAAGAAACCGCTTTCACTTACGCGATCACTTCTGGTGGAGTTGTCCAGTCCA 540
 103  S  N  A  S  P  D  K  I  I  A  V  G  S  K  E  T  A  F  T  Y  A  I  T  S  G  G  V  V  Q  S   132
 541  TTGCTCGCGCATGCAAAAGCGGAAATCTGATGGCGTGTGGCTGCAGTAAAAGAGAAAGACCCACGGGCCTGGGAAAAGACTGGAACTGGG 630
 133  I  A  R  A  C  K  S  G  N  L  M  A  C  G  C  S  K  R  E  R  P  T  G  L  G  K  D  W  N  W   162
 631  GAGGATGCGGCGACGATATCGATTATGCGTACGGATTTGCCCACGAGTTCATCGACGCTCAGGAACGCGACAATTCTTCTCCAAACGATC 720
 163  G  G  C  G  D  D  I  D  Y  A  Y  G  F  A  H  E  F  I  D  A  Q  E  R  D  N  S  S  P  N  D   192
 721  GCAGAGTGAAGTCTCACAAGGCAATGAATATTCATAACAACGAAGCGGGCAGACTGAGCGTTGTTCGCGCATCCCATACCACATGCAAAT 810
 193  R  R  V  K  S  H  K  A  M  N  I  H  N  N  E  A  G  R  L  S  V  V  R  A  S  H  T  T  C  K   222
 811  GCCACGGAGTATCGGGATCGTGTAGTATCAAGACTTGCTGGTTGCAAACGCCCCAGTTTCGGACTATAGGAGACAAATTGCGCCAGCGCT 900
 223  C  H  G  V  S  G  S  C  S  I  K  T  C  W  L  Q  T  P  Q  F  R  T  I  G  D  K  L  R  Q  R   252
 901  ACGACGACGCTCTTGAAATGCGAGTGACACATCGCGGACAAATGAAAACGAGATTTTCGTCCGATAGAAATCCTTCCAACATAGATCTCG 990
 252  Y  D  D  A  L  E  M  R  V  T  H  R  G  Q  M  K  T  R  F  S  S  D  R  N  P  S  N  I  D  L   282
 991  TATACATAGATTCTTCACCAGACTATTGCAAGGTCAATCACAAACTAGGGATATTAGGGACCTCCGGACGAGAGTGTCAACTCGATTCCC 1080
 283  V  Y  I  D  S  S  P  D  Y  C  K  V  N  H  K  L  G  I  L  G  T  S  G  R  E  C  Q  L  D  S   312
1081  TTGCTATGGACGGATGCGGTCTGATGTGCTGTGGGCGTGGGTACACAACGAAGATGGTGGAAGTAGTAAAATCGTGTAACTGTAAATTTC 1170
 313  L  A  M  D  G  C  G  L  M  C  C  G  R  G  Y  T  T  K  M  V  E  V  V  K  S  C  N  C  K  F   342
1171  AATGGTGCTGCTTTGTCAAATGTCAACAATGCAAAGAGAAGGTGCTGAAACACATTTGCAATTGATGGCAGCAGAAAACCTAACGGGTTC 1260
 343  Q  W  C  C  F  V  K  C  Q  Q  C  K  E  K  V  L  K  H  I  C  N  *                          363
1261  CTTATTGCAACATGTGTTGCCTTGAACCCCGTGATTTGAGTGTTCCATGCCTAATTGCTCGAATGCAACGTTTAGGTGAAATGTTCCGAG 1350
1351  GAAGGGAATTTTAGCCACGAAAGTGTGCTAATATTTCTCAAGTCGATTTTCCTGTTAGACCTCTCCAGACTAGATCAGTGTACACATATA 1440
1441  TATTTTATATTATTGATAGCAAAGTACATACCATGAAGTTTTAAAGCTTACCTCAAAAAATCCTTTATACAGAATGGGTTGTTGATGGTC 1530
1531  ACATTTTGAATGAACAGAACACAATTTATATTGAGAGGGGTCAAATAGTTATTGAGCTCAACCCATGATGGTTGTTTTGTAGAAATTATT 1620
1621  TTTACTTGGAATATAGCAGTATATTACTATCATTTTTTACGGGTTTGTTGAATGCGCCTATTTAACAATACTTAAATATGGTAATAATTG 1710
1711  TATTGCATTCCATGTGTTTTATAGCATCATTTGAAATGTTGCGTTTTAATGAAAATTCGTGTTTATACTATCACTTTTTATTAATTAGGA 1800
1801  TATCATTTCCCTAAATTTCACCCAATGTAAAAATGTAATGCAAATTGCGATTGCTCCTCGCTTCACTTTGGTAGGGGCTTATATTCTGCT 1890
1891  ATCGTGAAATTATCCATCGGCAGTATATAAATAGTATATAAAGGCTAAAAGTCTACATAAAATAGATAATACTGACATTGAGTTTATTCT 1980
1981  AGCATATGATACTATTTTTTACACTCCACTACTTTTAAGCACTTCACTCCCCCACGTTTCCTCCGCATGGATGCATCACCTTCTGATTTC 2070
2071  GTAGCACCAGAATGGTTTTTGTTGTTGTAATTTTTGTTTCAAAGCGTGTACCACCAGCACATAGAATAAATCATTATTTATTTATTGCCA 2160
2161  TAGCAAGGAACGGAGTAACCGATTCGTACAAGTCACTACACTATAGTTCTTGCAAGAGAGACGAAATTAACTGATTGTTATAAACTTTCG 2250
2251  TGGTCAATGACTTGTACTTTCTCACTGTAAATATTTTGTTGTGTGTTTTCTAACTCTTTAATATATTTTATATTTATGAAGGAAAGCTTT 2340
2341  TTGTTACTGATAATAAATTGAACGTTTGTATTAAC  2375
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B

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HrWnt-5     1  ---------------MVGMTRIQSAEPVWILFVLTLYSSVLMQVKPQLWSVGIERKKLFGETN-TSVH--CDEIRGLSRNQRSLCRTYNDH  73
ZfWnt-5     1  ------------------MDVRMNQGHLLLAVTLIVCNSQLLVVANSWWSLAM-NPIQRPEMYIIGAQPLCSQLTGLSQGQRKLCQLYQDH  72
XWnt-5A     1  MRKNLWTFQFGGEASGLVGSAMVSQHFVVLLMSLYCLTQSVVESSSWWSLGM-NPVQMPEVYIIGAQPLCSQLSGLSQGQKKLCQLYQDH  89
XWnt-5C     1  ----------------------MTPILRLLLLSSLLSCWKQSVVGANSWWSMAL-NPVQRPEMFIIGAQPLCSQLTGLSPGQRKLCQLYQDH  69
MWnt-5A     1  -MKKPIGILSPGVALGTAGGAMSSKFFLMALATFFSFAQVVIEANSWWSLGMNNPVQMSEVYIIGAQPLCSQLAGLSQGQKKLCHLYQDH  89
MWnt-5B     1  --------MLVPGHWDGLRPAMPSLLLVVVAALLSSWAQLLTDANSWWSLAL-NPVQRPEMFIIGAQPVCSQLPGLSPGQRKLCQLYQEH  81
                                                          **        *       *     *** *  ** *  *  *

HrWnt-5    74  MYYVESGSKQGVEECQWQFRGQRWNCSLASNASP-DKIIAVGSKETAFTYAITSGGVVQSIARACKSGNLMACGCSKRERPTGLGKDWNW  162
ZfWnt-5    73  MVYIGEGAKTGIKECQYQFRQRRWNCSTVDNTSVFGRVMHIGSRETAFTYAVSAAGVVNAVSRACREGELSTCGCSRAARPRDLPRDWLW  162
XWnt-5A    90  MQFIGDGAKTGIKECQYQFRHRRWNCSTVDNTSVFGRVMQIGSRETAFTYAISAAGVVNAVSRACREGELSTCGCSRAARPKDLPRDWLW  179
XWnt-5C    70  MVHIGEGAKTGIKECQHQFKHRRWNCSTVDNNSVFGRVMQIGSREASFTYAISSAGVVNAISRACREGELSTCGCSRTPRPKDLPRDWLW  159
MWnt-5A    90  MQYIGEGAKTGIKECQYQFRHRRWNCSTVDNTSVFGRVMQIGSRETAFTYAVSAAGVVNAMSRACREGELSTCGCSR-ARPKDLPRDWLW  178
MWnt-5B    82  MSYIGEGAKTGIRECQHQFRQRRWNCSTVDNTSVFGRVMQIGSRETAFTYAVSAAGVVNAISRACREGELSTCGCSRAARPKDLPRDWLW  171
               *    * * *  *** **   *****   * *        ** *  ****    ***    ***  * *  ****   **  *  ** *

HrWnt-5   163  GGCGDDIDYAYGFAHEFIDAQERDNSSPNDRRVKSHKAMNIHNNEAGRLSVVRASHTTCKCHGVSGSCSIKTCWLQTPQFRTIGDKLRQR  252
ZfWnt-5   163  GGCGDNVNYGYRFAREFVDARERENKYPRGSVEHARTLMNLQNNEAGRMAVYNLANVACKCHGVSGSCSLKTCWLQLADFRRVGEFLKEK  252
XWnt-5A   180  GGCGDNLDYGYRFAKEFVDARERENKIHQKGSYESSRIMMNLHNNEAGRRAVSTLADVACKCHGVSGSCSLKTCWLQLADFRKVGDHLKEK  269
XWnt-5C   160  GGCGDNVEYGYRFAKEFVDARERENKFPPKGSEEQARSLMNLQNNEAGRRAVYKLADVACKCHGVSGSCSLKTCWLQLADFRKVGEYIKEK  249
MWnt-5A   179  GGCGDNIDYGHPFAKEFVDARERERIHAKGSYESARILMNLHNNEAGRRTVYNLADVACKCHGVSGSCSLKTCWLQLADFRKVGDALKEK  268
MWnt-5B   172  GGCGDNVEYGYRFAKEFVDARERENKFAKGSEEQGRALMNLQNNEAGRRAVYKMADVACKCHGVSGSCSLKTCWLQLAEFRKVGDRLKEK  261
               *****   *  ** ** ** **  **  ******  *       ********** ******  **  *

HrWnt-5   253  YDDALEMRVTHRGQMKTRFSSDRNPSNIDLVYIDSSPDYCKVNHKLGILGTSGRECQLDSLAMDGCGLMCCGRGYTTKMVEVVKSCNCKF  342
ZfWnt-5   253  YDSAAAMRINRRGKLELVNNRFNPPTGEDLVYIDPSPDYCLRNETTGSLGTQGRLCNKTSEGMDGCELMCCGRGYDQFKTYKHERCHCKP  342
XWnt-5A   270  YDSAGAMKLNTRGKLVQVNNKFNSPTMNDLVYIDPSPDYCVHNESTGSLGTQGRLCNKTSEGMDGCELMCCGRGYDQFKTVQTERCHCKF  359
XWnt-5C   250  YDSAASMRLNKRNKLEQVNQRFNPPTGEDLVYLDPSPDYCLYNETTGSLGTHGRQCNKTSEGMDGCELMCCGRGYDQFKTVQVERCHCKF  339
MWnt-5A   269  YDSAAAMRLNSRGKLVQVNSRFNSPTTQDLVYIDPSPDYCVRNESTGSLGTQGRLCNKTSEGMDGCELMCCGRGYDQFKTVQTERCHCKF  358
MWnt-5B   262  YDSAAAMRITRQGKLELANSRFNQPTPEDLVYVDPSPDYCLRNETTGSLGTQGRLCNKTSEGMDGCELMCCGRGYDRFKSVQVERCHCRF  351
               ** * *                *  **** * ***** *  * *** ** *  *  **** ********          * * *

HrWnt-5   343  QWCCFVKCQQCKEKVLKHICN                                                                  363
ZfWnt-5   343  HWCCYVKCKRCTSLVDQFVCK                                                                  363
XWnt-5A   360  HWCCYVKCKKCTEVVDQFACK                                                                  380
XWnt-5C   340  QWCCFVKCKKCTEIVDQFVCK                                                                  360
MWnt-5A   359  HWCCYVKCKKCTEIVDQFVCK                                                                  379
MWnt-5B   352  HWCCFVRCKKCTEVVDQYVCK                                                                  372
               *** * * *  *   *
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Fig. 1. Nucleotide and amino acid sequences of *HrWnt-5*. (A) *The nucleotide and predicted amino acid sequences of a maternal cDNA clone HrWnt-5. The stop codon at termination site and those preceding the putative translation initiation sites are indicated by asterisk marks (*). The potential polyadenylation signal sequence is underlined. A poly(A) tail is not shown in this figure. The single open reading frame encodes a polypeptide of 363 amino acids. The N-linked glycosylation sites are shown by double underline. The N-terminus of the protein is characterized by a 27-amino acid-long hydrophobic region that contains a predicted signal sequence. The nucleotide sequence data reported here appears in the DDBJ, EMBL and GenBank Nucleotide Sequence Databases with the accession number (AB006608).* **(B)** *A comparison of the amino acid sequence of the HrWnt-5 with those of zebrafish Wnt-5 (Blader et al., 1996), Xenopus Wnt-5A (Moon et al., 1993a), Xenopus Wnt-5C (GenBank accession number 313267), murine Wnt-5A and -5B (Gavin et al., 1990). Identical residues are marked by asterisk marks(*). For maximal similarity, gaps were introduced.*

Wnts. Studies on several different types of secreted molecules have shed light on the mechanisms underlying axis formation and cell specification in embryos (Melton, 1991; Jessel and Melton, 1992; Moon *et al.*, 1997). Among these secreted molecules, *Wnts* are a family of structurally related cell-communication molecules encoding cysteine-rich, secreted glycoproteins (Moon, 1993; Moon *et al.*, 1997). In *Xenopus*, three members of the family including *XWnt-5A* (Moon *et al.*, 1993a), *XWnt-11* (Ku and Melton, 1993) and *XWnt-8b* (Cui *et al.*, 1995) are deposited in eggs as maternal transcripts; their biological functions as well as the precise spatial expression patterns in early embryos are not yet known. In the zebrafish, *ZfWnt5* is maternally deposited in the egg, although it is ubiquitously expressed in cleavage stage embryos (Blader *et al.*, 1996). Sequence analysis suggested that *HrWnt-5* belongs to the *Wnt-5* subclass. Whole-mount *in situ* hybridization revealed that maternally stored *HrWnt-5* transcripts are sequestered in the posterior-vegetal cytoplasm and concentrated in the posteriormost region of the embryos, while zygotically derived *HrWnt-5* transcripts are first observed in some vegetal blastomeres at the early 64-cell stage, then seen in a variety of cells in the entire embryo at the gastrula stage, and finally restricted to notochord cells in the tail in the tailbud stage embryo.

Results

Structure of HrWnt-5

Maternally expressed ascidian *Wnt* cDNA was isolated through the process of a sequencing strategy of randomly selected maternal cDNAs of *Halocynthia roretzi* (one out of 1,000 clones so far sequenced), following which the full length cDNA was cloned by screening a conventional cDNA library at high stringency condition using the primary cDNA as a probe. The longest clone recovered contains a 2.4 kb insert that includes all of the coding region as well as 143bp of 5' untranslated sequences, and 1143bp of 3' untranslated sequences (Fig. 1A). The predicted amino acid sequence from the open reading frame shown in Figure 1B, aligned with the sequences of some other known *Wnt* family members, contains 363 amino acids, including a hydrophobic signal sequence at the 5' end and all 24 of the invariant cysteine residues that are conserved among most of the other known *Wnt* genes and are diagnostic for WNT proteins. There were two putative glycosylation sites in this sequence.

To determine the *Wnt* subclass to which this gene belongs, we constructed a molecular phylogenetic tree. The tree shown in Figure 2 was calculated by the neighbor-joining method (see Materials and Methods). The tree suggested that this gene is a member of the *Wnt-5* subclass, as supported by a high bootstrap value (98%). We designated this gene *HrWnt-5*. The tree also showed that *HrWnt-5* is located at the root of further divergence of vertebrate *Wnt-5A*, *Wnt-5B* and *Wnt-5C*. In other words, *HrWnt-5* showed no special relationship with any *Wnt-5* subclass members. It is consistent with the previous report (Sidow, 1992).

To verify whether *HrWnt-5* is a single gene in the *Halocynthia* genome, genomic Southern blotting was carried out. As shown in Figure 3A, it is suggested that the ascidian genome contains only a single gene of the *Wnt-5* subclass.

A Northern blot analysis showed that *HrWnt-5* transcripts were present as a single mRNA species of 2.4 kb in length throughout embryogenesis, from unfertilized eggs to swimming larvae (Fig. 3B). Hybridization signals were detected in unfertilized eggs and in 16-cell stage embryos, indicating the maternally derived transcripts. Stronger signals were seen from the 64-cell stage embryos to neurulae, and then the signals gradually decreased to the undetectable level by the end of the larval stage, probably corresponding to the rise and fall of the zygotic transcription of the gene.

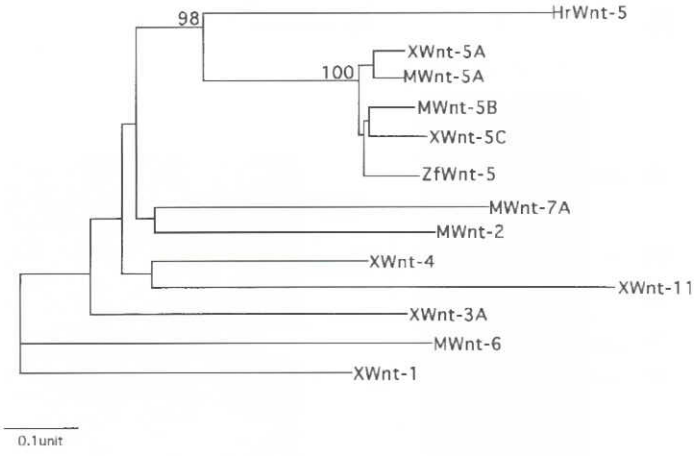

Fig. 2. Molecular phylogenetic tree of *Wnt* family genes. *This tree was made by the neighbor-joining method (Saitou and Nei, 1987). Bar indicates an evolutionary distance of 0.1 amino acid substitutions per position. The numbers at the node represent bootstrap value (%) for the grouping.*

Maternally derived HrWnt-5 mRNA is sequestered in the posterior-vegetal region of cleaving embryos

The localization of *HrWnt-5* maternal transcripts in the embryo was investigated by the whole-mount *in situ* hybridization of digoxigenin-labeled riboprobes for staged embryos. The transcripts were detected in the peripheral cytoplasm except for the animal pole region in unfertilized eggs (Fig. 4A). After fertilization, ooplasmic segregation occurs. Signals were detected in the subequatorial region in the eggs after the second phase of ooplasmic segregation (Fig. 4B), which relocates the peripheral cytoplasm in the unfertilized egg to the posterior-vegetal cytoplasmic domain called the myoplasm. The distribution of *HrWnt-5* transcripts formed a crescent-like structure corresponding to the myoplasm. During the early cleavage stages, the transcripts were sequestered in the posterior-vegetal region in the embryo. The animal pole view of the 2-cell-stage embryo shown in Figure 4C demonstrated the localization the transcripts in the narrow peripheral posterior cytoplasm in a bilateral manner. This localization was also observed in the 4-cell embryo (Fig. 4D). At the 8-cell stage, when the blastomere difference along the animal-vegetal axis is first generated, *HrWnt-5* transcripts were restricted to the posterior region of a pair of posterior vegetal blastomeres called B4.1 cells (Fig. 4E).

The sequestration of the transcripts continued as development proceeded. At the 16-cell stage, *HrWnt-5* transcripts were detected only in a pair of B5.2 cells, the posteriormost blastomeres of the embryo (Fig. 4F), while the myoplasm distributed to both daughter cells of B4.1 (B5.1 and B5.2). The signals were found in a pair of B6.3 cells of the 32-cell embryo (Fig. 4G), then in a pair of B7.6 cells of the 64-cell embryo (Fig. 4I). From the gastrula stage, the cells in which the transcripts were stored seemed not to divide further. The

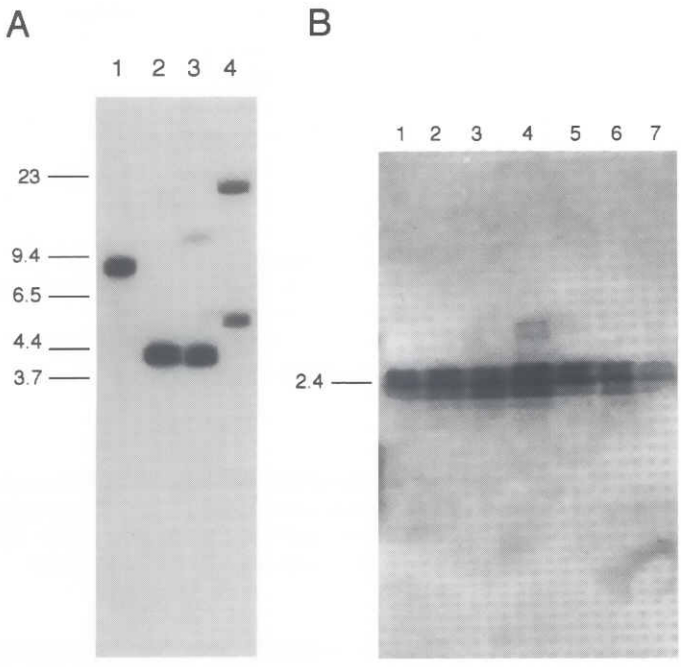

Fig. 3. Genomic southern and northern blot analyses of *HrWnt5*. (A) *Genomic southern blot analysis of HrWnt-5 gene. Genomic DNA isolated from a single adult was digested separately by BamHI (lane 1), EcoRI (lane 2), HindIII (lane 3), and PstI (lane 4). Ten micrograms of digested genomic DNA were loaded per lane. The blots were hybridized with ^{32}P-labeled DNA probes and the membrane was washed under high stringency condition. The numbers indicate size (in kb) of signals.* **(B)** *Temporal expression of HrWnt-5. Northern blots of poly(A)$^+$RNA prepared from unfertilized eggs (lane 1), 16-cell embryos (lane 2), 64-cell embryos (lane 3), gastrulae (lane 4), neurulae (lane 5), tailbud embryos (lane 6), and swimming larvae (lane 7) were hybridized with ^{32}P-labeled DNA probes and the membrane was washed under high stringency condition. Each lane was loaded with eight micrograms of poly(A)$^+$RNA. HrWnt-5 transcripts of about 2.4 kb in length were detected.*

signal was found in the position of the offspring of B7.6 cells expected from a cell lineage analysis (Nishida, 1987). Consistent with this observation, the transcripts were finally localized in a few cells of the ventral region of a tailbud, probably endodermal strand cells (Fig. 4P).

Expression of zygotic HrWnt-5 mRNA

The whole-mount *in situ* hybridization revealed that zygotic *HrWnt-5* transcripts are also expressed in the embryo. In ascidian embryos, hybridization signals of zygotic transcripts first appear in the nuclei (Makabe *et al.*, 1990). We found that there were no *HrWnt-5* signals in any nuclei until the 32-cell stage (Fig. 4G). At the early 64-cell stage, zygotic transcripts were first detected in A7.6, B7.1, B7.3 and a little later A7.1 cells (Fig. 4I). These cells give rise to trunk lateral cells (TLCs), endoderm, mesenchyme/secondary notochord and endoderm, respectively (Nishida, 1987). Then, at the middle 64-cell stage, A7.2 and A7.5 cells (Fig. 4J), which are primary notochord precursors, started to express the gene while the signals also became detectable in cells of the posterior epidermis lineage in the animal hemisphere, b7.14, b7.13, b7.11, b7.15 and b7.16 cells (Fig. 4H). At the late 64-cell stage, the signals at the vegetal region became stronger (Fig. 4K). At the 76-cell stage, B8.7 and B8.8, which are muscle precursors, started to express the gene, and the transcripts were widely distributed in the entire B-line blastomeres (Fig. 4M). Strong signals were still detected in TLC precursors and weak signals were noted in the primary notochord precursors. From the 76- to 110-cell stage, signals spread to the entire animal blastomeres with a gradient expression in strength from the maximal level posteriorly to a low level anteriorly (Fig. 4N). As shown in Figure 4N, at the 110-cell stage, the expression in A7.1, A7.2, B7.1, B7.2 and A7.6 cells disappeared while that in B7.5 and B7.7 cells remained strong, although these cells also give rise mainly to endoderm and mesenchyme. The signals in cells such as B8.15, B8.7 and B8.8 (muscle precursors) also remained strong. In addition, A8.15 and A8.16 cells, which are spinal cord precursors, showed strong staining. The signals in the primary notochord precursors became weaker and sometimes undetectable, while those in the secondary notochord precursors became stronger.

As shown in Figure 4Q, the signals began weaker at the top of the embryos from gastrula stage. The disappearance of the signals spread all over the embryo during the neurula stage except in the notochord precursors (data not shown).

In the tailbud stage, zygotic transcripts were seen exclusively in notochord cells (Fig. 4R). The primary notochord cells derived from A-line blastomeres had a signal intensity lower than that of the secondary notochord cells derived from B-line blastomeres (Fig. 4S).

Discussion

In the present study, we examined the sequence and expression of an ascidian maternal *Wnt* family member, *HrWnt-5*. A sequence analysis revealed that this gene contains all 24 cysteines and two of the four glycosylation sites conserved among *Wnt-5* subfamily members.

The phylogenetic tree also suggests that an ancestral *Wnt-5*, a vertebrate counterpart of *HrWnt-5*, duplicated during chordate evolution and diversified into *Wnt-5A*, *Wnt-5B*, and *Wnt-5C*. Gene duplication is thought to have occurred in many developmentally expressed genes, such as *Hox* cluster genes (Holland *et al.*, 1994) and myogenic factors (Atchley *et al.*, 1994), and is considered to be one of the major genetic changes that permitted the evolution from invertebrates to vertebrates (Holland *et al.*, 1994). Because *HrWnt-5* is located at the root of the branching of the vertebrate *Wnt-5* family, *HrWnt-5* may contain basic components and functions of the vertebrate *Wnt-5* family.

Sequestered maternal HrWnt-5 mRNA in the early ascidian embryos

We have shown that maternal *HrWnt-5* transcripts are present in *Halocynthia* fertilized eggs. During the early cleavage stages, the localized domain of the transcript overlaps with the myoplasm, which is thought to contain determinants for muscle cell differentiation (Nishida, 1992) and for the formation of the anterior-posterior axis (Nishida, 1994b). The localized domain, however, becomes smaller than an area of presumptive muscle cells as development proceeds. This suggests that the *HrWnt-5* transcripts are not the determinants of muscle cell differentiation.

The spatial distribution of the maternal *HrWnt-5* is exactly the same as that of *pem* RNA cloned from *Ciona savignyi* (Yoshida *et al.*, 1996). *pem* was shown to have a role in the establishment of the anterior and dorsal patterning of the embryo. The pattern of

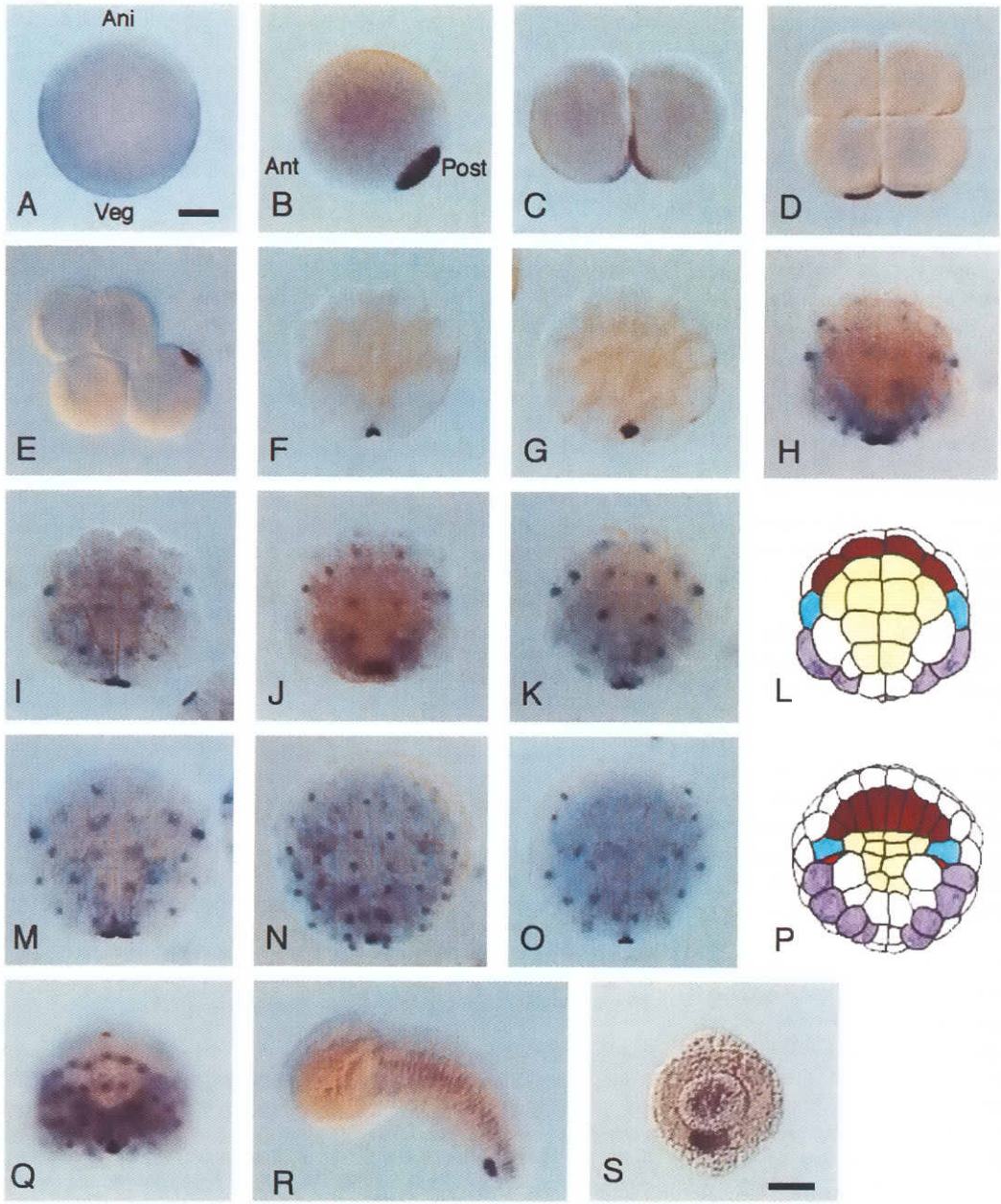

Fig. 4. Temporal and spatial expression of *HrWnt-5*, as revealed by whole-mount *in situ* hybridization. (A) *An unfertilized egg. ani, animal pole; veg, vegetal pole of the egg. The animal pole is determined by DAPI staining (data not shown).* **(B)** *A fertilized egg after completion of the second phase of ooplasmic segregation. ant, anterior; post, posterior. The A-P axis is determined by DAPI staining (data not shown).* **(C)** *A 2-cell embryo.* **(D)** *A 4-cell embryo.* **(E)** *A 8-cell embryo, lateral view.* **(F)** *A 16-cell embryo, vegetal view.* **(G)** *A 32-cell embryo, vegetal view.* **(H)** *A 64-cell embryo, animal view.* **(I)** *An early 64-cell embryo, vegetal view.* **(J)** *A middle 64-cell embryo, vegetal view.* **(K)** *A late 64-cell embryo, vegetal view.* **(L)** *A diagram of a 64-cell embryo, vegetal view. Each color represents notochord (red), endoderm (yellow), trunk lateral cell (blue), and muscle (purple).* **(M)** *A 76-cell embryo, vegetal view.* **(N)** *A 110-cell embryo, animal view.* **(O)** *A 110-cell embryo, vegetal view.* **(P)** *A diagram of a 110-cell embryo, vegetal view.* **(Q)** *A middle gastrula, posterior view.* **(R)** *A tailbud embryo, lateral view.* **(S)** *A cross-section through a tail region of an early tailbud embryo stained for HrWnt-5. Signals are detected in the endodermal strand and notochord. Bar, 50 μm.*

HrWnt-5 distribution, together with the molecular nature of the *Wnt* family members, is thus, rather consistent with the possibility that *HrWnt-5* may be involved in the establishment of the axes in the ascidian embryo.

3'UTR plays an important role in the localization of maternal transcripts in *Drosophila* (reviewed by Ding and Lipshitz, 1993) and in *Xenopus* (e.g., Mowry and Melton, 1992). Sequence comparisons of the *HrWnt-5* and *Ciona pem* 3'UTR do not show any obvious similarity. There is, however, still a possibility that the same RNA localization machinery works in both cases. Analyses of maternal genes in *Ciona savignyi* recently revealed several mRNA species also sequestered in the same region as *pem* RNA (Satou and Satoh, 1997; Yoshida *et al.*, 1997). These results lead us to a hypothesis that an unknown cytoplasmic region containing specific molecules such as *Wnt* RNA is present in the posterior-vegetal cytoplasm in the ascidian embryo; we would name it 'postplasm'. Postplasm is distinguished from myoplasm as early as the 16-cell stage, at which myoplasm distributes to two daughter blastomeres of B4.1, while postplasm is inherited only by the posterior daughter blastomere. Interestingly, an electron microscopic study of the 8-cell ascidian embryo revealed an electrodense structure in the region in which a centrosome-attracting body exists (Iseto and Nishida, 1996). This may be a core structure of a multifunctional complex anchoring the specific RNAs in postplasm.

There are two possibilities on how maternal *HrWnt-5* mRNA is sequestered in the postplasm: the mRNA is transferred in mass from the myoplasm to the postplasm or it is degraded in the myoplasm but not the postplasm. Both possibilities cannot be excluded now.

Expression domain of zygotic HrWnt-5 mRNA

HrWnt-5 is also one of a few zygotic ascidian genes that are expressed not in a lineage-dependent manner but rather in a region-dependent manner. In particular, the expression of *HrWnt-5* in animal blastomeres seems to form a gradient of the transcripts, which should probably result in the graded concentration of the signaling protein products. This may be achieved by successive interactions of neighboring epidermal cells. Murine and chick *Wnt-5A* are known to display a gradient in the limb bud (Dealy *et al.*, 1993; Parr *et al.*, 1993). It has been hypothesized that the graded expression of *Wnt-5A* may be important for the development of the three proximodistal segments of the limb; however, this is not the case in the ascidian embryo, in which no segmental structure is observed in the *HrWnt-5*-positive area.

The spatial expression pattern of the zygotic *HrWnt-5* transcripts in the vegetal hemisphere is most complex from the 64-cell stage to the neurula stage, around a period of morphogenetic movements for gastrulation and neurulation. There is convincing evidence that *Wnts* play a role in the control of cell adhesion (e.g., Moon *et al.*, 1993b; Torres *et al.*, 1996). This observation suggests that *HrWnt-5* may regulate cell adhesion, cell shape, and the morphogenetic movements of the embryo.

In the tailbud stage, zygotic *HrWnt-5* transcripts were seen only in notochord cells in the present study. This is an expression pattern not observed in any vertebrates, and the present findings are the first report of this pattern in notochord cells. The role of *HrWnt-5* product in notochord cells is unknown. The expression levels of *HrWnt-5* in primary and secondary notochord cells are not equal; a low level was observed in anterior primary notochord cells and a high level was detected in posterior secondary notochord cells. There are some differences reported between the notochord cells of these two lineages (Whittaker, 1990; Nakatani *et al.*, 1996; Tanaka *et al.*, 1996). Notochord cells of different origin may have different functions to form a body.

The developmental functions of *HrWnt-5* remain to be determined. Products of *Wnt* family members may activate a receptor-mediated signal transduction pathway leading to changes in the morphogenetic movements of tissues and/or the regulation of cell fates. In addition to the classical signaling pathway, it is shown that *XWnt-5A* may activate a phosphatidylinositol signaling pathway via heteromeric G-protein subunits (Slusarski *et al.*, 1997). The clarification of the functions of *HrWnt-5* must be carried out based on its spatial patterns of expression. The expression pattern of this gene shows that *HrWnt-5* is involved in a variety of processes during embryogenesis. The expression patterns of *HrWnt-5* in ascidians is markedly different from that observed in vertebrates. However, the developmental function of a particular gene is not necessarily directly assessed by the conservation of expression among different organisms. Rather, the diversified developmental strategies in different organisms can be investigated by utilizing the differences in the expression patterns of homologous genes. An overexpression study using a microinjection technique into ascidian eggs is now in progress to elucidate the developmental functions of *HrWnt-5*.

Materials and Methods

Animals and embryos

H. roretzi was purchased during the spawning season from fishermen near the Otsuchi Marine Research Center, Ocean Research Institute, University of Tokyo, Iwate, Japan. *H. roretzi* is hermaphroditic and self-sterile. Naturally spawned eggs were fertilized with a suspension of non-self sperm. When fertilized eggs were cultured at about 12°C, they developed into gastrulae and early tailbud embryos about 12 h and 24 h after fertilization, respectively.

Eggs and embryos at appropriate stages were packed by low-speed centrifugation and frozen with chilled ethanol for Northern blotting or fixed for *in situ* hybridization.

Sequence analysis of HrWnt-5

A full-length cDNA was obtained by screening a cDNA library of fertilized eggs using an original partial cDNA as a probe. The cDNA was cloned into the plasmid vector pBluescript, and was used as a template for sequencing by an automated DNA sequencer (ABI PRISM 377, Perkin Elmer Japan, Chiba).

The amino acid sequences of the *Wnt* family gene products were aligned and gaps were introduced for maximal similarity; 288 confidently aligned sites were then analyzed. The molecular phylogenetic relationships of the *Wnt* family gene products were estimated by means of neighbor-joining (Saitou and Nei, 1987) using the PHYLIP version 3.5c computer software package (Felsenstein, 1993). The distance matrix was constructed according to the Dayhoff model (Dayhoff *et al.*, 1978). Confidence in the phylogeny was assessed by bootstrap resampling of the data (Felsenstein, 1985).

Isolation of nucleic acids and Southern/Northern blotting

Genomic DNA was isolated from a gonad of a single adult using formamide (Sambrook *et al.*, 1989). Total RNA was extracted using AGPC (Chomczynski and Sacchi, 1987). Poly(A)$^+$RNA was purified using Oligotex-dT30 beads (Roche Japan, Tokyo). Filter hybridization was performed by standard procedures (Sambrook *et al.*, 1989) with a ^{32}P-labeled DNA probe, and membranes were washed under high stringency conditions.

In situ hybridization

Whole-mount specimens were hybridized *in situ* at 42°C using digoxigenin-labeled antisense probes, as described by Miya *et al.* (1994). After visualization of the hybridization, the embryos were dehydrated and rendered transparent with a 1:2 mixture (v/v) of benzyl alcohol and benzyl benzoate. Some embryos were embedded in polyester wax and sectioned at 8 μm to confirm the localization of hybridization signals in the embryo.

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