

## [SHORT COMMUNICATION]

## Trunk Lateral Cell-Specific Genes of the Ascidian *Halocynthia roretzi*

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**ABSTRACT**—Cell lineage analysis of the ascidian *Halocynthia roretzi* demonstrated that a pair of cells situated on the right and left sides of 64-cell stage embryos, termed the A7.6 cells, give rise to trunk lateral cells (TLCs) of the tadpole larva and that after metamorphosis TLCs give rise to various mesodermal tissues of the adult, in particular all of the blood cells or coelomic cells. Here we report the isolation and characterization of cDNA clones for two TLC-specific genes *HrTLC1* and *HrTLC2*. *HrTLC1* encodes for a novel protein while *HrTLC2* encodes for a polypeptide with RNA recognition motifs. Zygotic expression of *HrTLC1* and *HrTLC2* begins at the neural plate stage and transcripts of both these genes are restricted to TLCs.

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### INTRODUCTION

Lineage analysis of embryonic cells of the ascidian *Halocynthia roretzi* by means of intracellular injection of horseradish peroxidase, demonstrated that a pair of right and left A7.6 cells of the 64-cell stage embryo give rise to a group of about 16 cells situated between the dorsolateral wall of the endoderm and the dorsolateral epidermis of the mid-tailbud stage embryo (Fig. 1A, B; Nishida, 1987). These cells were named “trunk lateral cells (TLCs)” because of their position within the embryo (Nishida, 1987). TLCs differ from mesenchyme cells and trunk ventral cells (TVCs).

By tracing the fates of TLCs using a specific antibody suggest that TLCs are the precursors of some types of adult blood cells (Nishida *et al.*, 1989). Further analyses of cell lineages from the embryonic (larval) stage to juvenile (adult) stage confirm this notion, namely that TLCs are the only source of coelomic cells (blood cells) of juveniles (Fig. 1C; Hirano and Nishida, 1997), although TLCs also give rise to adult muscle cells and cells within the gill-slits (Fig. 1C; Hirano and Nishida, 1997). These results clearly indicate that although TLCs are likely to have several roles during embryogenesis, they have important roles in the formation of the adult body. In particular, blood cells of ascidians have attracted researchers because of their various functions, including immune responses, vanadium accumulation and the formation of asexual

buds in colonial ascidians (see a review by Satoh, 1994). Therefore, it is an intriguing question to ask what kinds of genes are expressed in TLCs and in the embryonic progenitor cells that differentiates into adult blood cells. To date, however, there are no reports that describe genes that are specifically expressed in TLCs of the ascidian embryo. Here we report the characterization of two TLC-specific genes of the *Halocynthia* embryo.

### MATERIALS AND METHODS

#### Biological materials

Naturally spawned eggs of the ascidian *Halocynthia roretzi* were fertilized with a suspension of sperm from another individual, and fertilized eggs were raised at 15°C. Embryogenesis proceeded synchronously among eggs of certain batches. They developed into gastrulae about 10 hr after fertilization and early tailbud embryos at 18 hr of development. Larvae hatched at about 35 hr after fertilization.

#### Isolation of cDNA clones and nucleotide sequencing

A cDNA library of the tail region of *H. roretzi* mid-tailbud-stage embryos and a library of the trunk region of the embryos were constructed in  $\lambda$ ZAP II (Stratagene; Takahashi *et al.*, 1997). Differential screening of the libraries with a total cDNA probe of the tail region yielded several positive clones for the tail region. Preliminary analyses of spatial expression of the genes by *in situ* hybridization of whole-mount specimens demonstrated that the transcripts of two clones are specific to TLCs (Takahashi *et al.*, 1997), and the corresponding genes were named *HrTLC1* and *HrTLC2*.

Sequences of the clones were determined for both strands with a Big-Dye Primer Cycle Sequencing Ready Reaction Kit and ABI-PRISM 377 DNA sequencer (Perkin Elmer).

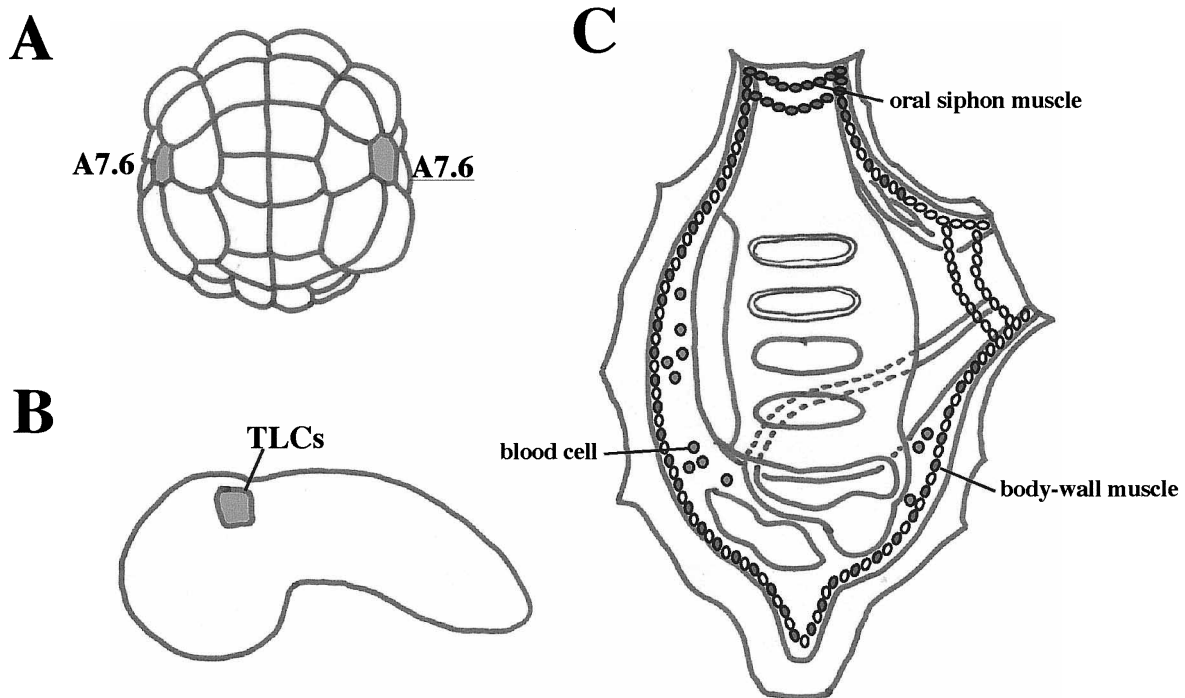
#### Whole-mount *in situ* hybridization

*In situ* hybridization with whole-mount specimens was carried

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**Fig. 1.** Illustration showing the cell lineage that gives rise to the trunk lateral cells (TLCs) in *H. roretzi* embryos and juveniles. (A) A 64-cell stage embryo, vegetal view; (B) tailbud embryo, lateral view; and (C) juvenile. TLCs (shown by darker shading) originate from the A7.6 pair of the 64-cell stage embryo, and give rise to mainly blood cells of juveniles [Based on Hirano and Nishida (1997)].

out using digoxigenin-labeled RNA probes as described previously (Takahashi *et al.*, 1997). In brief, embryos at appropriate stages were fixed in 4% paraformaldehyde in MOPS buffer (pH 7.8), 0.2 M NaCl, 0.4 M MgCl<sub>2</sub>. After a thorough wash with PBT [phosphate-buffered saline (PBS) containing 0.1% Tween 20], the fixed specimens were treated with 2 µg/ml proteinase K (Merck) in PBT for 30 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 hybridized with the digoxigenin-labeled antisense or sense probe for at least 16 hr at 42°C. The probes were synthesized from the coding region of the gene by following the instructions from the supplier of the kit (DIG RNA Labeling kit; Boehringer Mannheim). After hybridization, the specimens were washed and treated with RNase A, and then they were washed extensively with PBT. The samples were then incubated for 1 hr with 1:2000 Boehringer Mannheim alkaline-phosphate-conjugated anti-DIG and treated for the development of color as indicated in the protocol from Boehringer.

## RESULTS

During experiments to isolate genes that are expressed either in the tail region or trunk region of *H. roretzi* tailbud embryos, we noticed that two cDNA clones were expressed only in TLCs (Takahashi *et al.*, 1997). The present study further characterized these cDNAs.

Fig. 2 A shows the nucleotide and deduced amino acid sequences of *HrTLC1* cDNA clone. The insert of the clone encompassed 2767 bp and contained a single open reading frame that predicted a polypeptide of 579 amino acids. The calculated relative molecular mass of the predicted protein was 65 kDa. A BlastX search demonstrated that HrTLC1 pro-

tein has neither special sequence motifs nor similarity to known proteins, although the protein resembled the C-terminus region of a *C. elegans* gene (H04M03.4) with an unknown function (Fig. 2B). As shown in Fig. 3A, *in situ* hybridization signals of *HrTLC1* zygotic transcripts were first detected at the neural plate stage. A few cells situated at the left and right sides of the prospective trunk region showed signals. At the tailbud stage, signals were evident in a pair of TLC masses (Fig. 3B, C). These signals persisted until the late tailbud stage, and no embryonic cells other than TLCs showed the hybridization signal.

Fig. 4 shows the nucleotide and deduced amino acid sequences of *HrTLC2* cDNA. The insert of the clone encompassed 3810 bp and contained a single open reading frame that predicted a polypeptide of 427 amino acids. The calculated relative molecular mass of the predicted protein was 46 kDa. The BlastX search demonstrated that the HrTLC2 protein contained two RNA binding motifs, KGYGFVDF at the position 131–138 and KGVGFARM at position 209–216. Amino acid sequences around these motifs were shared by two reported proteins, one is a chicken *c-myc* gene single-strand binding protein-1 (MSSP-1; Negishi *et al.*, 1994; Kimura *et al.*, 1998) and a *C. elegans* R10E4.2b gene product. The chicken MSSP-1 was composed of 373 amino acids and possessed two RNA binding domains (Fig. 4), which were responsible for binding to RNA and ssDNA, respectively (Negishi *et al.*, 1994). Although MSSP-1 mRNA is distributed ubiquitously, MSSP-1 suppresses transcription of  $\alpha$ -smooth muscle actin gene in chicken visceral smooth muscle cells

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1 ATTTAAATAGATAGTTGGCAATATTTGCATTTGAACGCGTACCTGTTGGAAGTATCTGTTGATTTACTTTTCATCTTGGAGATTTCTAACG 90
91 TGAACCTGTGACGTCATATATCAATCATGGGAAACGCACCTCGAGGAAACGACGGGTCAGGCGGACGTTATGATTATCGGAGGCGGACCCA 180
1 M G N A L E G T T G G Q A D V M I I G G G P T 22
181 CAGGCATCGGGGAGCTTACAGACTCAATCAATATGGACALCAACAAATTTGGTTCCTTTTGGACGCAGAGAAACAACAGGTGGCCTTGCTG 270
23 G I G A A Y R L N Q Y G H N N W F L L D A E K Q P G G L A G 52
271 GCTCGATTAAACTCAGGAAGGATTCGGTTCGATCATGGATACAAAACGTTTTTTAGTCGTTACAAATTTCTGGATTTGTCTGATGGATG 360
53 S I K T H E G F R F D H G Y K T F F S R Y K F L D C L M D D 82
361 ATTTGCATTCGAAAGCGTCTCCTCGCAAACCTTATGAAAAGAGTCGAGACAACACTACGTTTTTCGTTAAGGGGCGTCTAGTACGTTATCCTC 450
83 C H S K A S P R K L M E K S R D N Y V F V K G R L V R Y P L 112
451 TGCAAAATAACCTTTCCGGATTGCGGAAAAGACCAAGAATCTTGGCGAGTTGATCTTCTTTGTTTCGAGGATTAGAACAAGGAAACAA 540
113 Q N N L S G L P E K D Q E S C A V D L L C S R I R N K E T K 142
541 AAAAAAGCAAGGACGAGGATAAAAAGAAATGAAGAGCAAATTTGATCAGACGGAAGAAAGGGAAGATATCACGAAAGAAAAGAAGATATCG 630
143 K A K D E D K K N E E Q I D Q T E E R E D I T K E K E D I E 172
631 AGCAACACAAGAGGAAAAGCAAGCAGCTGTGAAATCCAAGATGGCGACTCTCATGTATCAGACGACAATACAGCCCGAGTGAAG 720
173 Q T Q E G K D E A A V E I Q D G D S H V S D D N T A P S E E 202
721 AAGGAAATGATTGTGAAGAAATAGAAAACATTAGACATTTAGTTTCGGAATTTGGGAGAAAGTCTTTGCAATATCTTATTTCTGCTCTT 810
203 G N D C E E I E T L D H Y L L R N W G E S L C N I L F R P Y 232
811 ACATTTTAAATCGTTCCGCTATCCATCCAGTAAGCTGACTTCGTCGTTGGGCTGAGTCAAAAATCCGCCTTCTATTTTAGGAGATGATA 900
233 I F K S F A Y P S I F K L T S S W A E S K I P S I L G D I 262
901 TTGAGAAGATTATAAGAGGGGAACAAAGCGATCCAGGACATGGAGCAGCAATCACTATCTATCCATCCAAAGGAGGACTCGTTCTTCT 990
263 E K I I R G E Q S D P G H G A D A I T I Y P S K G G L V S F 292
991 TTTGGAGAACAATAGTGGCAAAATACCTTAAAGAAAACGTTTCGCTTTTAAAGCGAAAATTTGTTGATCTGGACATCGAGCAAAAACATCC 1080
293 W R T I V R K L P K E N V R F K S E I V D L D I E Q K L I R 322
1081 GGACCAAGAGCGGACTGAAAATAAAAATACAACACTCTGATAAGCACCATGCGGATCACGTCCTGCGGATTTAGCAGGAGGGAGGATC 1170
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1171 TTGGGAGTCGTTAGCTCATTTCATCTTTACATATTTGTGTGTTGGAAATAAGAGCGGTGAGCACAATGAAAGATATAAAAGGATGCATTT 1260
353 G E S L A H S S L H I V C L L G I R G V S T M K D I K G C I Y 382
1261 ACTATCCTGAATCCGATTGCATATCCATCGCGCATGTGATTTCCAGTTTTCGATCCGGATAGCATCCAGACAAAGATCAAAGATCC 1350
383 Y P E S D C I F H R A C V F S S F D P D S I P D K D Q K I P 412
1351 CGACTCTACGTTTTGCTAAACCTTCGGAAGACGGCAATGAGATACGAGGAAGTTCAGTACACCGGTCGGTATTTGGTTCCTTTGCTTTTGG 1440
413 T L R F A K P S E D G N E I R G S S S H T G P Y W S L L L E 442
1441 AAATAAGTGGAGGACCACTGAAAACAATTCACGACGCGACAATAATGGACGAGGTTATACGTGACGCTGTGCAGTGGGTTTGTCTGAGAC 1530
443 I S G G P L K T I H D A T I M D E V I R D A C A V G L L R P 472
1531 CAGACGCAAAATCGTTTCAATACACATGACAGAGATGACACATGGATTCCTTACCACGCTGGAGAGTGGAGATTGAGATTGATAAAG 1620
473 D D E I V S I H M T E M T H G Y P L P T L E S E I E I D K A 502
1621 CACTAAATTTGGCTCAAAGTAAGGGGATCTATAGTCGAGGGCGGTTTGGTTCCTTTCAAATACCACGTGGGAGACTTGGACCAATGTCTGA 1710
503 L N W L Q S K G I Y S R G R F S F K Y H V G D L D Q C L I 532
1711 TCCAAGGGTGGAGCTGTGCAACATATAATAAAGGAGAACCCGAGAAATGCATCTGGAGCAAAGGAAATGAAATATCCGACCGAAGAAA 1800
533 Q G V E A V D N I I K G E P E K C I W S K G N E I S S E E R 562
1801 GATACCTTACCCGCTATATAATCTGGAACCCATCCGGAAGAGGAAAGCCAGCTAATAAACCGAGGCGCAATAAGGAGTGGCATAACAAG 1890
563 Y L T P S I I W N P S R K R K A S * 579
1891 AGAACCTCTAACCGTGTGGGCTCGAGCACAACATCTGTTACCACGCTCTTCTGCAACCTCTGATGCAGGACAATTAAGTGATTCGGAAC 1980
1981 AGGGAGCTCTGTATCGGGTAATACAAGCTAAGGAATGGATTTTCACATTTCAACCTTGATCCCAAATCTTGACACTATTTTTTGGATC 2070
2071 CTGCATTTGGGGAATCAAATAGTGTATGCTTTACAGGCTATTATTTAGTCTGTAGAAATGTCCAAGCCGGAAGTCGCAACACATTTCCCA 2160
2161 TAACACATATGAAAAAATGTGCAAAATTTTTCAGTATAGAAATGTGAAAACCTTTCTGTGCAAAATTAATAATTTAAAATTCGTTTGATAC 2250
2251 TCCTGCATTTGGGAAATCTAAATAGTGTATGCTTTACAGGCTATTATTTATGCTGTAGAAATGTCCAAGCCGAAAGTTCGCAACACATTTCC 2340
2341 CATAACACATATAAAAAAATGTGCAAAATTTTTCAGTATAGAAATGTGAAAACCTTTCTTCAAATTAATAATCAAATTCGTTTGAT 2430
2431 AATTCTAAGCAATATGTGGTTCAATCAACAAGGGACAAATTTTTTCACTCGTCAATTTGTACCATATCTACTATTTATTTTTTATATG 2520
2521 TAATATATAATACATACAGTATCAAAATACCTTGTATCAAAATCTTGTCTTATAATTCACACCTATCACTAAGACACATTTTTTACAA 2610
2611 CATGTATATCTTTGTGTTTTATGTGTCTTCAATTCGTTGTTCTTCTTATTCAGCGCTTTGGTTATCTCTTTCCACTCTTTTCTGTGT 2700
2701 ATTAATAAATGGAATGCAATAGCAGTACAACATGCTTTGCAGTATCACAAAAAATAAAAAAAAAA 2767
    
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**B**

HrTLC1	487	GYPLPTLESEI	EIDKALNWL	QSKG	YSRGRF	SFKY	HVGDLD	QCL	LOGV	AVDNI	KGEPEK	548	
HO4MO3.4	412	GYEIP	PNRDRE	LARAHG	ELRHQ	YSRGRF	GWKY	EASNQD	HCF	LOGKE	FIDRVIL	GEPEK	473

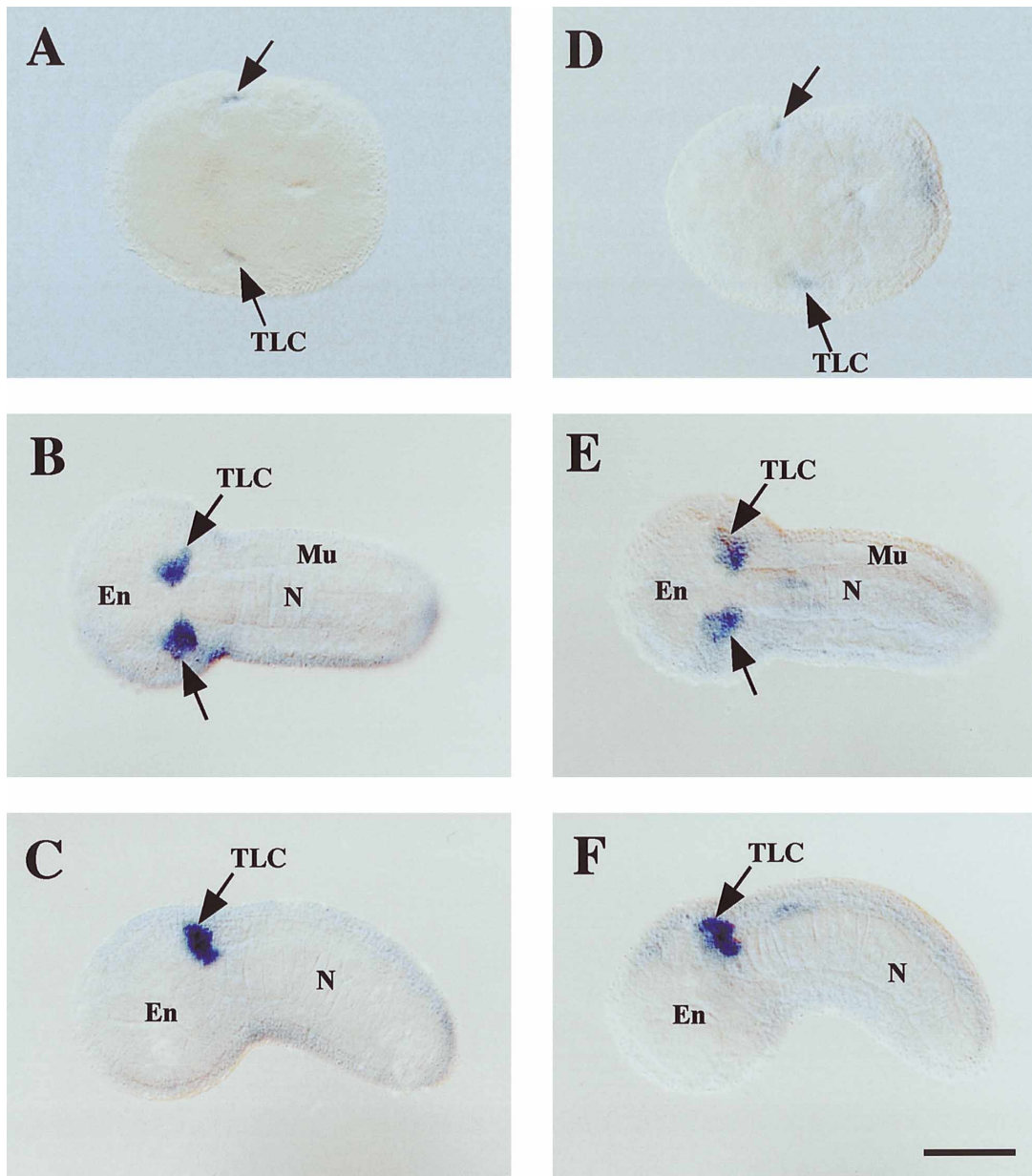
**Fig. 2.** (A) Nucleotide and predicted amino acid sequences of the cDNA clone for the *HrTLC1* gene. The ATG at the position 117–119 represents the putative start codon of the *HrTLC1*- encoded protein. A potential signal sequence for polyadenylation is underlined. An asterisk indicates the termination codon. The nucleotide sequence will appear in the DDBJ/EMBL/GenBank Nucleotide Sequence Databases with the accession number (AB053356). (B) Alignment of amino acid residues of HrTLC1 with those of *C. elegans* gene product (HO4MO3.4) with unknown function. Identities between them are boxed.

(Kimura *et al.*, 1998).

Similar to *HrTLC1* mRNA, *in situ* hybridization signals of *HrTLC2* were first detected at the neurula stage (Fig. 3D) and they were evident in two masses of TLC (Fig. 3E, F). A few cells in the dorsal nerve cord also showed weak hybridization signals (Fig. 3E, F).

**DISCUSSION**

An ascidian tadpole larva contains a number of pockets of mesenchyme cells (Conklin, 1905; Katz, 1983). It is estimated that around the time of hatching the ascidian larva contains about 900 mesenchyme cells. In addition, trunk lateral cells (Nishida, 1987) and trunk ventral cells (Whittaker, 1990) represent distinct subpopulation of mesenchyme cells. Previ-



**Fig. 3.** Spatial distribution of zygotic transcripts of *HrTLC1* (A–C) and *HrTLC2* (D–F) in *H. roretzi* embryos. Anterior of the embryo is to the left. (A, D) Embryos at the neural plate stage, dorsal view. Cells showing the expression of these genes are indicated by arrows. (B, C, E, F) Early tailbud embryos, dorsal view (B, E) and lateral view (C, F). Arrows show TLCs with distinct hybridization signals. En, endoderm; Mu, muscle; and N, notochord. Scale bars = 100  $\mu$ m.

ously it was thought that larval mesenchyme cells formed various mesodermal tissues of the adult, including blood cells, body wall muscle and heart (reviewed by Satoh, 1994). However, Hirano and Nishida (1997) clearly showed that *H. roretzi* mesenchyme cells give rise only to tunic cells, and that TLCs give rise to blood cells, longitudinal mantle muscle, oral siphon muscle, and ciliary epithelium of the 1st and 2nd gill slits (Fig. 1C). On the other hand, TVCs form mantle and siphon muscle and heart (Hirano and Nishida, 1997). In particular, TLCs are the only embryonic source of adult blood cells. Therefore, *HrTLC1* and *HrTLC2* are genes that are expressed exclusively in blood precursor cells.

Cellular mechanisms involved in the specification of TLCs have been studied with a TLC-specific monoclonal antibody (Nishikata and Satoh, 1991; Kawaminami and Nishida, 1997). Recent experiments involving the isolation and recombination of blastomeres at the 16-cell stage showed that an inductive influence emanating from cells within the animal hemisphere (presumptive epidermal blastomeres) is required for TLC formation (Kawaminami and Nishida, 1997). This inductive activity is distributed widely within the animal hemisphere. By contrast, only presumptive TLC blastomeres have the competence to be induced to form TLCs. The cDNAs isolated in the present study provide valuable probes for further

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1 CATATATCTATATCCCTCTATATACTTTATATAGCCTGTATAGCCTAATCAGTATTTAATCTATTTCGTAACAAAAAATTAACATTAAA 90  
91 AATATTAGAGTACCCCTTCATAACCGTCCAACCTCCGGTGAATCTATATCCATTCCCTTGTGTATCTTGTGTATATTTGATTTCAAACCT 180  
181 ATCGTATCCAAACACTCCCTAAGTTGGAAACCAACGAAATCACCAGTCCAAGACCAACGAAAAACGAGGTTTCGAACACAGTATTTCTAC 270  
271 CCTGCGTAGCTATTGTGTATATTTGCTTGTAAAATCTGCTCCGCGGTGTAAACATCCGTCATACGTGTGTTCAAGATAAACAGTTGTT 360  
361 GAATAACAGCATGTACTACTGGGCATCTAACCCCGTTCAGTTTGGCCATACGCTCACGCAACTGCCAGCACCACGCTCGCAACATGCC 450  
1 M Y Y W A S N P V P V W P Y A H A T A Q H H A R N M P 27  
451 GCCCCGCTCCATCCATCGCCAGTAGCACCTCCTCTCTCAACATTCACAGTACAGGTAGTCCCTCAAGCGGCTCTGGTAGTCTCTCA 540  
28 P P S P S I A S S T S S S S Q H S N S T G S P S S G S G S P Q 57  
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58 N M N L Q N N S A N Q Q S S G M N G H S Q M N G Q G D L 87  
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88 S K T N L Y I R G L T P N T S D E D L V N L C K Q Y G K I V 117  
721 ATCCACCAAGGCTATCATCGATCCACAAACCAGCCCTTTGTAAGGATATGGATTTCGCGATTTCGATCGCTATGAGTCCGCTGTCCCTTGC 810  
118 S T K A I I D P Q T S L C [K G Y G F V D F] D R Y E S A V L A 147  
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148 V T S L K G T K A Q A Q M A K Q Q E Q D P T N L Y L S G L P 177  
901 ACCTAATTTCAATGAGCAGACTTAGAACAAATGCTCTCTTATGGTCAAGTATCTTACCAGAACTCCTTCGCGACAGCAATGGTGT 990  
178 P N F N E H D L E Q M L L P Y G Q V I S T R I L R D S N G V 207  
991 CAGCAAGGAGTAGGATTTGCCAGAAATGGAATCAAAGGAAAAATGGCAAGTCAATATCTCAAAGTTTAATGGAAAAATATCTAACGGGAGG 1080  
208 S [K G V G F A R M] E S K E K C E V I I S K F N G K Y L T G G 237  
1081 CAACACCCACAGTACTGAGCCCTTATTGTGCAAGTTTGTCTGATGGAGGACCAAGAAAAACAGCAACATCAGAAGTTTATGGCTAATGG 1170  
238 N T H S T E P L L C K F A D G G C G P K K N K Q H Q K F M A N G 267  
1171 ACGGACCTGGAGGATGGCGAAATGCCTTTTCCTTATGACACCCCGCCCTGACCCATAATGGGATGGCTCCCAATCGCATGATGATGCA 1260  
268 R T W R D G G E M P F P Y D P A A L T H N G M A P N R I M M Q 297  
1261 GCCATACCTAACAGGGCAACTAATCCCGCTTACCAGTGCAAGCCCAACATCGGATGCGAGCTCCTCCTTATGTTGTACAGCCTCA 1350  
298 P Y L T G P T N P A Y Q M Q A C P P T W M Q P P Y V Q P H 327  
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328 V T T S T V I S S M D P N M H C P S V M P S V M P Q L S Q L T A 357  
1441 CCAGATGACACAGCTTCAACTCTACCAAAATCCAGGAGCCATTTTCGTACGGACCAAAATGGTGTGTGTCGGCCATACATCCCAAACTA 1530  
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1621 ACAAGCGACAGTGAAGAACAGTATAGCACATAATCGGAAAAAGTGCTTTTGGAAATCCGCAATGTTGTGATGATTGAGTCAATCAATA 1710  
418 Q A T S E E Q Y S T \* 427  
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3781 GCAAGCAACGCAAAAAA AAAAAAAAAA 3810

B

HrTLC2	88	S	K	N	L	I	R	G	L	T	P	N	T	S	D	E	L	V	N	L	C	K	Q	Y	G	K	I	V	175
MSSP-1	27	S	X	I	N	L	I	R	A	L	P	P	N	T	D	D	L	V	K	L	C	P	Y	G	K	I	V	S	114
R10E4.2b	95	S	S	I	N	L	I	R	G	L	M	P	N	T	N	D	L	R	E	M	C	S	K	Y	N	I	A	S	194

HrTLC2	176	L	P	P	N	F	N	E	H	D	L	E	Q	M	L	L	P	Y	G	Q	V	I	S	T	R	I	L	R	D	S	N	G	V	231	
MSSP-1	115	L	P	L	S	M	D	E	Q	E	L	N	M	K	P	F	G	Q	V	I	S	T	R	I	L	R	D	S	S	G	T	S	R	170	
R10E4.2b	195	L	P	L	D	F	T	Q	M	L	E	N	K	F	G	V	I	S	T	R	I	L	R	D	S	N	G	V	S	K	E	K	E	V	250

Fig. 4. (A) Nucleotide and predicted amino acid sequences of a cDNA clone for the *HrTLC2* gene. The ATG at position 371–373 represents the putative start codon of the *HrTLC2*- encoded protein. A potential signal sequence for polyadenylation is underlined. An asterisk indicates the termination codon. Amino acid sequences of two RNA-recognition motifs are boxed. The nucleotide sequence will appear in the DDBJ/EMBL/GenBank Nucleotide Sequence Databases with the accession number (AB053355). (B) Alignment of amino acid residues of the RNA recognition motifs of HrTLC2, MSSP-1 and *C. elegans* R10E4.2b gene product. Identities are boxed.

analysis of molecular mechanisms underlying TLC specification.

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