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Kyoto University
**[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.

**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 (Nishide 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 differentiate 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 λZAP 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 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 out...
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 MgCl2. 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 protein 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 α-smooth muscle actin gene in chicken visceral smooth muscle cells.
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. Previous studies have shown that the expression of specific genes in these cells is crucial for the development of the ascidian tadpole larva. The identification of novel genes encoding proteins with unknown functions could provide new insights into the molecular mechanisms that govern ascidian tadpole development.
Previously 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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**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 µm.
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.
analysis of molecular mechanisms underlying TLC specifica-

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REFERENCES


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