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Monoclonal Antibodies against Differentiating Mesenchyme Cells in Larvae of the Ascidian *Halocynthia roretzi*

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ABSTRACT—Mechanisms of cell specification of mesenchyme during ascidian embryogenesis are poorly understood. This is because no good molecular markers have been available to evaluate differentiation of the mesenchyme cells. To obtain molecular markers of mesenchyme differentiation, we established monoclonal antibodies, Mch-1 and Mch-3, that recognize antigens present in the mesenchyme cells of the larva of *Halocynthia roretzi*. The antigens recognized by both antibodies start to be detectable in the mesenchyme cells at the late tailbud stage. The Mch-3 antibody specifically recognized all mesenchyme cells of the larva, whereas the Mch-1 antibody stained the cells only in the anterior portions of mesenchyme clusters in the trunk region of the larva. The Mch-1 antibody also stained trunk lateral cells. In addition, both antibodies recognized the mesenchyme cells in the ventro-lateral boundary between endoderm and epidermis that are migrating to the anterior head region of the larva. The partial embryos that originated from the mesenchyme-lineage cells at the 8-cell stage expressed the Mch-1 and Mch-3 antigens. The Mch-1 and Mch-3 antibodies will be useful as immunological probes for studying the specification mechanisms of mesenchyme cells.

INTRODUCTION

Ascidian embryos develop into tadpole-shaped swimming larvae, which have a primitive chordate body plan. The larvae have remarkable features, such as a muscular tail, a notochord and a dorsal neural tube, and based on this, the ascidians are classified along with vertebrates in the phylum Chordata (Kowalevsky, 1866; Drach, 1948; Katz, 1983). The larva has a very simple body plan. The tadpole larva is comprised of only a few distinct types of tissue (Katz, 1983). The major tissues are epidermis, nerve cord, brain (sensory vesicle), endoderm, muscle, notochord and mesenchyme. The simple organization of the larva facilitates studies on the mechanisms of cell specification in all the tissues of the embryo. Furthermore, the cleavage pattern and the developmental fate of each blastomere are invariant among individual embryos. The cell lineage during embryogenesis was described in detail and has now been well established (Conklin, 1905; Ortolani, 1955; Nishida, 1987; Nicol and Meinertzhagen, 1988). Thus, the ascidian is a good experimental animal to explore cell specification mechanisms.

Mechanisms of cell specification for most of the tissues in the ascidian embryo have been revealed at the cellular level (for reviews, see Satoh, 1994; Nishida, 1997). The developmental fates of epidermis, endoderm and muscle are determined by tissue determinants localized within egg cytoplasm (Conklin, 1905; Reverberi and Minganti, 1946; Nishida, 1992, 1993, 1994). By contrast, the developmental fates of notochord and brain are determined by cellular interactions (Rose, 1939; Reverberi *et al*., 1960; Okado and Takahashi, 1988; Nishida, 1991; Nakatani and Nishida, 1994). However, mechanisms of cell specification of mesenchyme and nerve cord are poorly understood. This is because no good molecular markers are available to evaluate differentiation of these kinds of tissues. Recently, Araki *et al*. (1996) isolated a cytoskeletal actin cDNA (*HrCA1*) from *Halocynthia*. The gene is specifically expressed in mesenchyme, nervous system and notochord cells. The mechanism of fate specification of mesenchyme cells was analyzed to some extent using the cDNA probe.

The term mesenchyme has been loosely used for a long time to describe embryonic cells that are found as individuals or in groups rather than in a sheet, and that have not differentiated into a specialized cell type (Davenport, 1895). In the trunk region of the ascidian tadpole larva, there are a number of mesenchyme cells (Fig. 1). Two mesenchyme clusters (bilateral pair) are found between the ventromedial endoderm and the ventrolateral epidermis in the trunk region of the *Halocynthia* larva (Fig. 1E, G and H). The mesenchyme cells are nearly spherical and smaller than the other tissue cells in the larva. The cytoplasm of the mesenchyme cells is less granular, and contains fewer yolk granules than the surrounding cells (Katz, 1983). It was reported that most of the mesenchyme cells give rise to tunic cells of the adult organism after metamorphosis (Hirano and Nishida, 1997).

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Ascidian embryos are bilaterally symmetrical. As shown in Fig. 1A, all mesenchyme cells are derived from the B4.1 blastomere pair of the 8-cell embryo. At the 110-cell stage, the B8.5 and B7.7 cell pairs are mesenchyme precursors (Fig. 1B). The fate of these cells is already restricted to give rise to mesenchyme cells (Nishida, 1987). These primordial mesen-

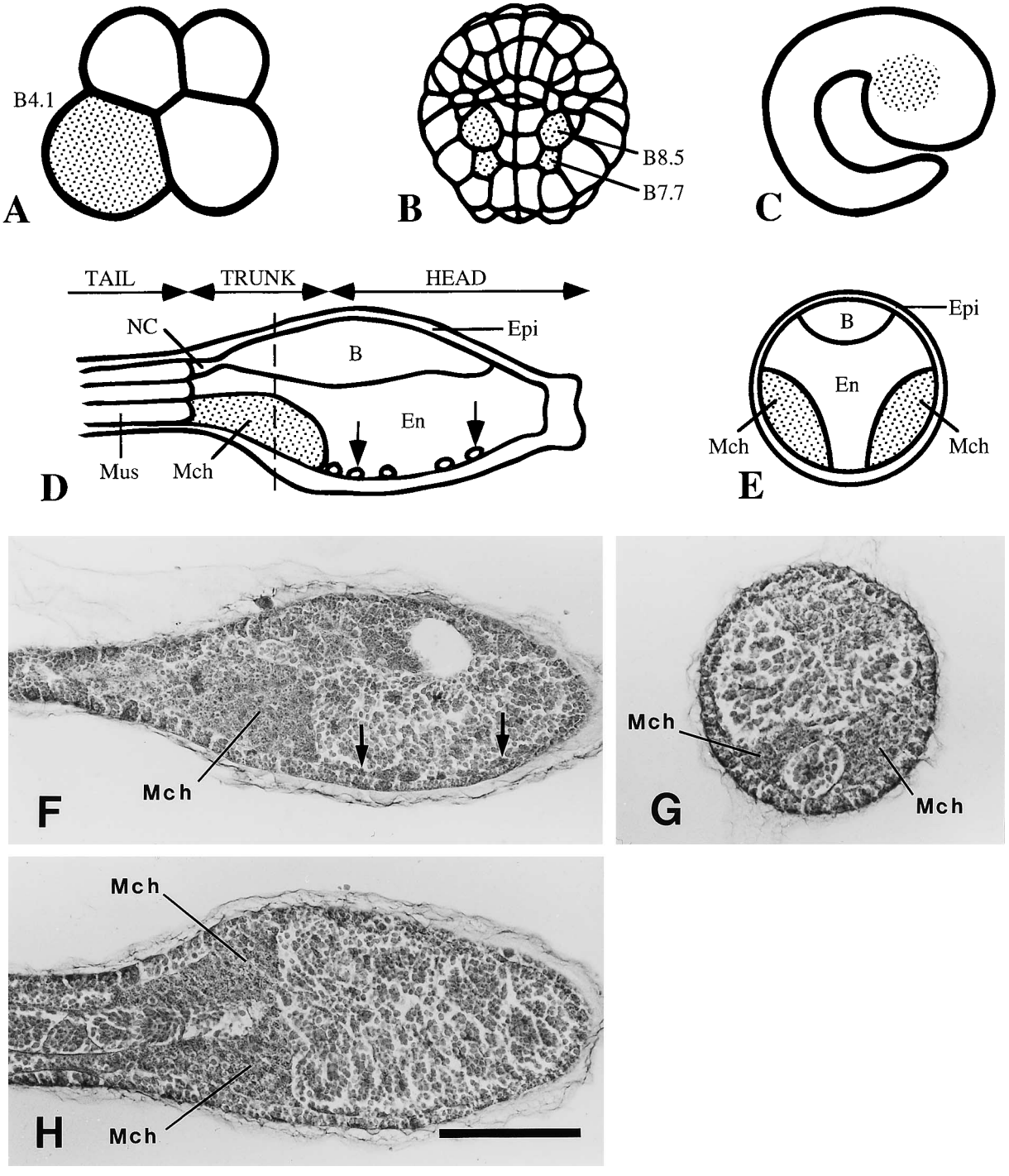

Fig. 1. Diagrams illustrating the cell lineage of mesenchyme cells (**A** - **C**) and the structures of tadpole larvae of *Halocynthia roretzi* (**D** and **E**). The positions of the blastomeres of the mesenchyme lineage in embryos and mesenchyme cells in larvae are indicated by shading. (**F** - **H**) Paraffin sections ordinary stained with hematoxylin-eosin. (**A**) The 8-cell embryo. Lateral view. Anterior is to the right. (**B**) Vegetal view of an embryo at the 110-cell stage. Anterior is up. The names of primordial mesenchyme cells are indicated. (**C**) Middle tailbud stage. (**D**) Parasagittal section of hatched larva. Some mesenchyme cells are migrating in the anterior direction through the ventro-lateral boundary between the endoderm and epidermis in the head region (arrows). Broken line marks the position of the transverse section in **E**. (**E**) Clusters of mesenchyme cells located on both sides of the trunk region. (**F** and **G**) Photographs of sections corresponding to **D** and **E**, respectively. (**H**) Frontal section. B, brain; En, endoderm; Epi, epidermis; Mch, mesenchyme; Mus, muscle; NC, nerve cord. Scale bar, 100 μm.

chyme blastomeres at the late blastula will divide at least seven or eight times before the hatching to produce several hundred mesenchyme cells (Satoh, 1994). To study the formation of mesenchyme cells, we have produced monoclonal antibodies that recognize differentiating mesenchyme cells in ascidian embryos.

MATERIALS AND METHODS

Embryos

Naturally spawned eggs (280 μm in diameter) of *Halocynthia roretzi* were artificially fertilized with a suspension of nonself sperm and raised in filtered seawater at about 13°C. At 13°C, tadpole larvae hatched about 35 hr after fertilization.

Production of monoclonal antibodies

Monoclonal antibodies were produced as described by Mita-Miyazawa *et al*. (1987). Larvae just before or after metamorphosis were collected and homogenized in ice-cold physiological saline solution (0.9% NaCl) on ice. The homogenate was centrifuged at about 10,000 × g for 5 min at 4°C to remove yolk granules. The supernatant was used as an antigen and injected intraperitoneally into female BALB/c mice several times. Three days after the last immunization, mouse spleen cells were fused with myeloma cells (PAI) using 50% polyethylene glycol 4,000 Mw (Merck). The hybridoma cells were cultured in HAT medium. Each clone was screened by indirect immunofluorescence as described below.

Fixation and immunofluorescence staining

Tadpole larvae were fixed with absolute methanol for 10 min at –20°C, then with cold absolute ethanol. Fixed larvae were embedded in polyester wax (Steedman, 1957; BDH Chem. Ltd., UK). To screen the hybridoma supernatant, embedded samples were sectioned at 6 μm and mounted on small coverslips. After removal of the polyester wax with ethanol, specimens were washed with phosphate-buffered saline. Indirect immunofluorescence staining with supernatant of hybridoma culture medium was carried out by standard methods using FITC-conjugated secondary antibody (Cappel, Ohio, USA).

Inhibition of cleavages

Cleavage of 110-cell stage embryos was permanently arrested with 2 μg/ml of cytochalasin B (Sigma). Under this condition, embryos immediately ceased cytokinesis but continued nuclear divisions (Whittaker, 1973). Cleavage-arrested embryos were cultured until control embryos reached the hatching stage. They were fixed and then devitellinated for immunofluorescence staining.

Isolation of blastomeres

Fertilized eggs were manually devitellinated with sharpened tungsten needles and reared in 0.9% agar-coated plastic dishes that contained filtered seawater. Identified blastomeres were isolated from 8-cell embryos with a fine glass needle under a stereomicroscope (SZH-10, Olympus). Isolated blastomeres were cultured separately as partial embryos until the hatching stage and fixed for immunofluorescence staining.

RESULTS

Production of monoclonal antibodies against mesenchyme cells

Homogenates of whole larvae were used as antigen because it is difficult to isolate and collect a large amount of pure mesenchyme cells from the larvae. Monoclonal antibody techniques were adopted because specific antibody can be generated using crude immunogen (Yelton and Scharff, 1981). We screened individual hybridoma culture fluid samples by immunofluorescence staining. Three hybridoma cell lines, 4A6 (Mch-1), 2A7 (Mch-2) and 1G11 (Mch-3), secreting antibodies that recognized differentiating mesenchyme cells were identified and cloned.

In this paper, we report characteristics of the Mch-1 and Mch-3 antibodies only, because the class of the Mch-2 antibody is IgM, and staining of mesenchyme cells was less evident when the whole mount larvae were stained with the Mch-2 antibody.

The Mch-3 antibody

The Mch-3 antibody specifically recognized all mesenchyme cells in hatched larvae. The antibody stained cells in the mesenchyme clusters in the trunk region (Fig. 2A - C). The Mch-3 antibody also recognized mesenchyme cells migrating through the ventro-lateral boundary between the endoderm and epidermis to the anterior head region (Fig. 2A and B, arrows). Mesenchyme cells seemed to migrate away from the anterior-lateral edge of the clusters (Fig. 2B, downward arrows). Morphology and size of these migrating mesenchyme cells are indistinguishable from mesenchyme cells in the clusters. Some mesenchyme cells were observed to appear in the anterior head region even before hatching.

To determine when the expression of the Mch-3 antigen first became detectable during embryogenesis, various staged embryos were stained with the antibody. The antigen started to be detected in the mesenchyme clusters at the late tailbud stage. The Mch-3 antibody stained small particles in mesenchyme cells. Larvae were double stained with the Mch-3 antibody and 0.1 μg/ml of DAPI, which is a fluorescent dye specific for DNA (Fig. 2D and E, respectively). The particles appeared to closely associate with nuclei, although it is not clear whether the particles lie inside or outside the nuclei.

The Mch-1 antibody

The Mch-1 antibody recognized a subset of mesenchyme cells. The antibody only stained cells in the anterior parts of mesenchyme clusters (Fig. 2F - J). Mesenchyme cells in the posterior parts of the clusters were not stained. This staining pattern was reproducibly observed. The Mch-1 antibody also recognized the migrating mesenchyme cells, as did the Mch-3 antibody (Fig. 2F and H, arrows).

In addition, the Mch-1 antibody stained cells (about 15 μm in diameter) larger than the mesenchyme cells (about 6-10 μm in diameter) (Fig. 2G and H, arrowheads). These cells were dispersed in relatively dorsal parts of the trunk and head regions of larvae. It seems that these larger stained cells in the dorso-lateral region are trunk lateral cells (TLCs). TLCs were first described as larval cells situated dorsally to mesenchyme cell clusters at the tailbud stage (Nishida, 1987). These cells differ from endoderm and mesenchyme cells. To confirm the staining of TLCs with the Mch-1 antibody, sections and whole mount specimens of larvae were stained with TLC-specific monoclonal antibody 5A4A3 (Mita-Miyazawa *et al*.,

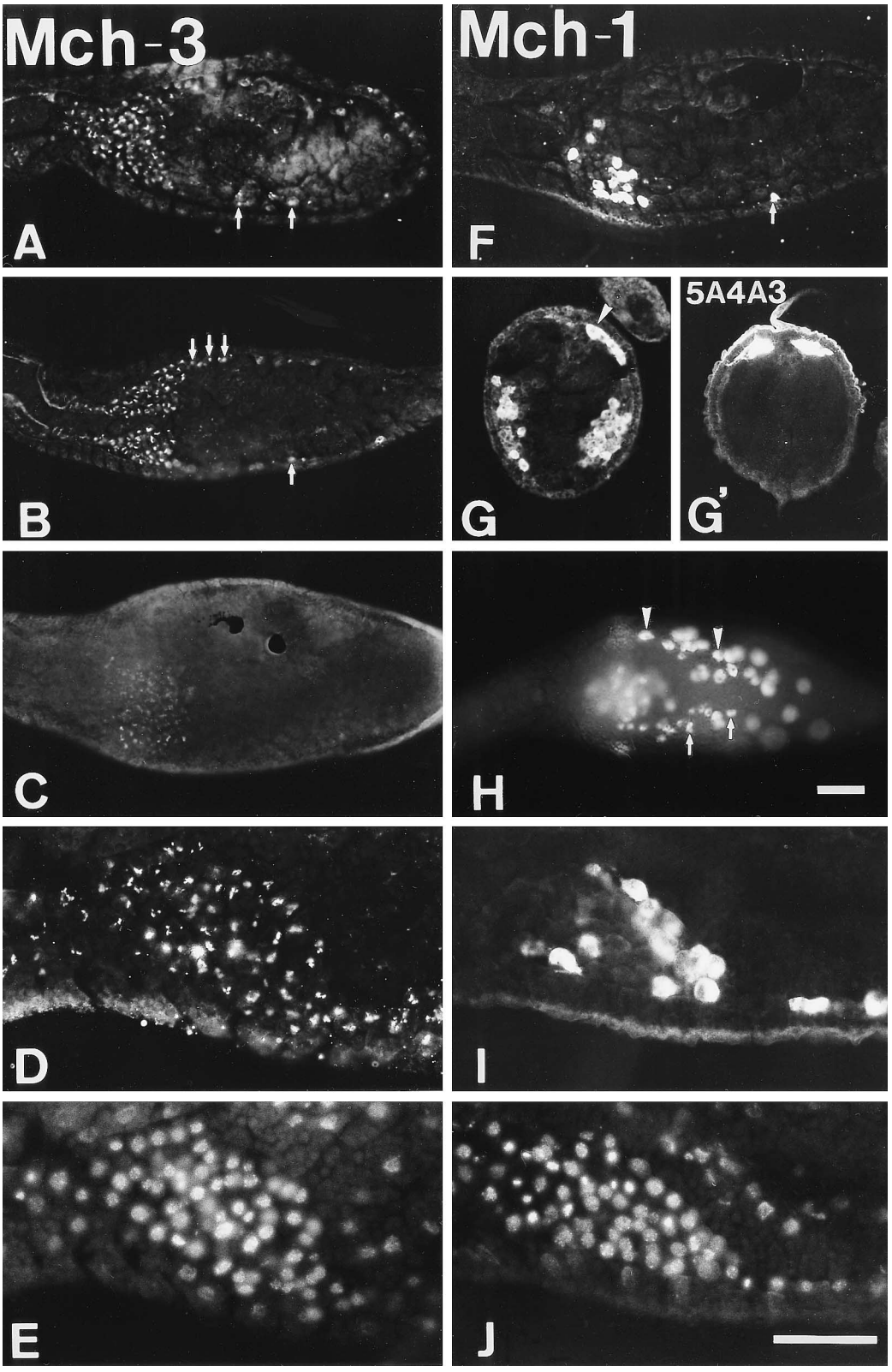

Fig. 2. Immunohistochemical staining of tadpole larvae with the Mch-3 (left panels) and Mch-1 (right panels) antibodies. (**A** and **F**) Parasagittal sections. (**B**) Frontal section. (**G** and **G'**) Transverse sections of the trunk region. (**C** and **H**) Whole mount staining. (**D** and **I**) Higher magnification of the stained cells. (**E** and **J**) Nuclear staining with DAPI of the same sections as **D** and **I**, respectively. (**A** - **E**) The Mch-3 antibody specifically recognized all mesenchyme cells of the clusters in the trunk region and migrating mesenchyme cells (arrows). (**F** - **J**) The Mch-1 antibody stained mesenchyme cells in the anterior portion of the mesenchyme cluster and migrating mesenchyme cells (arrows), as well as larger cells, which are TLCs, in the dorsal part (arrowheads). (**G'**) Section similar to **G** is stained with TLC-specific antibody (5A4A3). Scale bars, 50 μm.

1987; Nishide *et al*., 1989), and the stained cells were compared to those stained with the Mch-1 antibody. The result indicated that the larger cells recognized by the Mch-1 antibody are TLCs because the same kind of larger cells was also recognized by the 5A4A3 antibody (Fig. 2G and G').

The initiation of expression of the Mch-1 antigen in TLCs preceded that in mesenchyme cells. The expression in TLCs started at the middle tailbud stage. The antigen recognized by the Mch-1 antibody started to be detected in the mesenchyme clusters at the late tailbud stage, similarly to the antigen of the Mch-3 antibody (data not shown). The Mch-1 antibody stained the whole cytoplasm of mesenchyme cells except the nuclei (Fig. 2I and J).

As shown in Fig. 1A, all mesenchyme cells are derived from the B4.1 blastomere pair of the 8-cell embryo. The fate to produce mesenchyme cells is inherited by the B7.7 and B8.5 blastomeres of the 110-cell embryo (Fig. 1B). The B8.5 blastomere pair gives rise to cells of the anterior part of the mesenchyme clusters, and mesenchyme cells in the posterior part of the clusters are derived from the B7.7 blastomere pair (Nishida, 1987). The Mch-1 antibody only stained mesenchyme cells in the anterior parts of the clusters. Therefore, there is a possibility that the Mch-1 antibody recognizes only the descendants of the B8.5 blastomeres. To examine this possibility, we observed the expression of the Mch-1 antigen in cleavage-arrested embryos, in which cleavage after the 110-cell stage was permanently inhibited by 2 μg/ml cytochalasin B. In ascidians, cleavage-arrested embryos often express various tissue-specific markers in the correct lineage precursor cells (Whittaker, 1973). As shown in Fig. 3A, expression of the Mch-1 antigen was found in the cleavage-arrested B8.5 blastomeres (in 5 out of 18 cases, large arrows), but no expression was observed in the B7.7 blastomeres (in 18 cases, small arrows) in the cleavage-arrested 110-cell embryos. The A7.6 blastomeres, the TLC precursors, were also stained (in 13 out of 18 cases, arrowheads). This result supports the idea that the Mch-1 antibody recognizes TLCs as well as mesenchyme cells that are derived from the B8.5 lineage, but not cells derived from the B7.7 lineage.

The Mch-1 and Mch-3 antibodies will be immunological probes for studying the mesenchyme specification

Experiments with blastomere isolation are essential to reveal autonomy and non-autonomy of tissue formation during ascidian embryogenesis and are the first step to study fate specification (Satoh, 1994; Nishida, 1997). To assess the suitability of Mch-1 and Mch-3 antibodies as molecular markers for differentiation of mesenchyme cells, we examined the expression of antigens recognized by these antibodies in the partial embryos derived from each isolated blastomere of 8-cell embryos (Table 1). In normal development, all mesenchyme cells originate from the B4.1 blastomeres (Fig. 1A). Reverberi and Minganti (1946) reported that B4.1 partial embryos constitute muscle, endoderm and mesenchyme cells using morphological criteria. Similarly, we observed that B4.1 partial embryos contained cells nearly spherical in shape and smaller than the other cells. These cells loosely attached to the partial embryos, and looked like mesenchyme cells. In 68 out of 73 cases (93%), the B4.1 partial embryos expressed the antigen that is recognized by the Mch-3 antibody (Fig. 3B and C). The Mch-1 antibody also stained some cells in the B4.1 partial embryos (Fig. 3D and E, in 41 out of 48 cases, 85%). Staining was observed in mesenchyme-like small cells. The antibodies stained none of the A4.1, a4.2 or b4.2 partial embryos (Table 1). TLCs are derived from the A4.1 blastomere pair, but the Mch-1 antibody did not stain most of the A4.1 partial embryos. This result is in accordance with the report by Kawaminami and Nishida (1997), in which it was shown that cellular interaction at the 16-cell stage is required for the differentiation of TLCs. Thus, the Mch-1 and Mch-3 antibod-

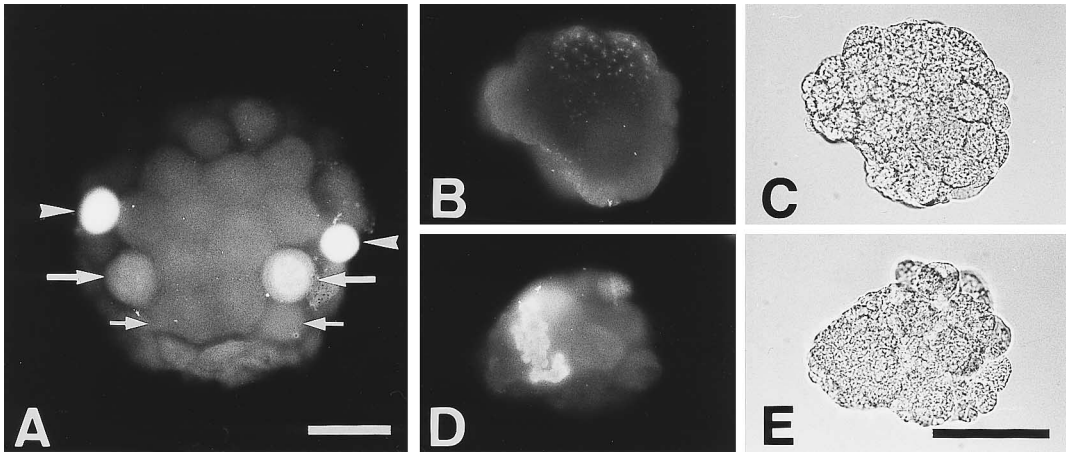

Fig. 3. (**A**) The expression of the Mch-1 antigen in cleavage-arrested 110-cell embryos was observed in the B8.5 blastomere pair (large arrows), but not in the B7.7 blastomere pair (small arrows). The antibody also stained the A7.6 blastomeres, which are precursors of the TLCs (arrowheads). (**B** and **D**) The expression of antigens recognized by the Mch-3 (**B**) and Mch-1 (**D**) antibodies in partial embryos that were derived from isolated B4.1 blastomeres of 8-cell embryos. (**C** and **E**) Morphology of the partial embryos corresponding to **B** and **D**, respectively. Scale bars, 100 μm.

Table 1. Expression of the Mch-1 and Mch-3 antigens in partial embryos derived from each blastomere of 8-cell embryos

Blastomeres	Partial embryos with the Mch-3 antigen/Examined embryos	Partial embryos with the Mch-1 antigen/Examined embryos
a4.2	0/44	0/41
b4.2	0/44	0/37
A4.1	0/36	1/32 (3%)[a]
B4.1	68/73 (93%)	41/48 (85%)

[a] A small number of cells that are of the same size as TLCs was stained only in one case.

ies will be used as molecular markers for monitoring the formation of mesenchyme cells in experiments with blastomere isolation.

DISCUSSION

The Mch-1 and Mch-3 monoclonal antibodies recognized mesenchyme cells in *Halocynthia* larvae. The Mch-3 antibody specifically recognized all mesenchyme cells of the larva. The Mch-1 antibody stained cells in anterior parts of the mesenchyme clusters in the trunk region. The results of cleavage-arrested experiments suggest that only the descendants of the B8.5-line mesenchyme precursors expressed the Mch-1 antigen. This staining pattern suggests that mesenchyme cells in the clusters are not homogeneous at the hatching stage.

Both antibodies recognized the cells migrating through the ventro-lateral boundary between endoderm and epidermis to the anterior head region. These cells are mesenchyme cells. Indeed, the mesenchyme cells were observed to migrate from the clusters to anterior head region by labeling mesenchyme cells with a lineage tracer, DiI (T. Hirano and H. Nishida, unpublished observation). In addition, anterior movements of mesenchyme cells were also observed in living larvae of *Ascidia ahodori*, which have relatively transparent larvae (H. Nishida, unpublished observation). The expression of the antigens was initiated at the late tailbud stage, and some mesenchyme cells were observed to start to migrate just prior to hatching. Although mesenchyme cells do not differentiate to their terminal state during larval development in *Halocynthia*, they initiate certain processes associated with differentiation during embryogenesis and before metamorphosis.

Mechanisms of fate determination of mesenchyme cells are not well understood. Reverberi and Minganti (1946) reported that the B4.1 blastomeres isolated from 8-cell embryos autonomously develop into partial embryos that have muscle, endoderm and mesenchyme cells. In their report, formation of mesenchyme cells was evaluated using morphological criteria of mesenchyme cells. In the present study, the B4.1 partial embryos expressed both the Mch-1 and Mch-3 antigens. This is consistent with the observation of Reverberi and Minganti (1946). Recently, Araki *et al*. (1996) reported that cell-cell contact before the 8-cell stage is not required for specification of mesenchyme cells in the ascidian embryo, using a cytoskeletal actin cDNA probe (*HrCA1*) as a molecular marker for mesenchyme formation.

However, the results of blastomere isolation at early stages do not exclude the possibility that cellular interactions occur within isolated partial embryos. The B4.1 blastomeres isolated from 8-cell embryos have the prospective fate to differentiate into cells other than mesenchyme. Non-mesenchyme cells, such as muscle and endoderm cells, might induce the differentiation of mesenchyme cells. Inductive interactions may occur within the partial embryos. Indeed, such an event was demonstrated by Nakatani and Nishida (1994). Notochord formation had been thought to be an autonomous process based on the results of blastomere isolation at early cleavage stages. However, recent experiments that involved detailed isolation and recombination of blastomeres at various cleavage stages demonstrated that notochord is induced at the 32-cell stage. Therefore, more detailed and careful experiments, such as continuous dissociation of embryonic cells as well as isolation of the precursor blastomeres at later stages, should be carried out to establish whether the development of mesenchyme cells is an autonomous process. It will be possible to investigate in detail the mechanisms involved in cell fate determination of mesenchyme cells using the Mch-1 and Mch-3 antibodies by molecular detection of mesenchyme formation.

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